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(54) Title: METHODS AND REAGENTS FOR THE RAPID AND EFFICIENT ISOLATION OF CIRCULATING CANCER CELLS

(57) Abstract

A highly sensitive assay is disclosed which combines immunomagnetic enrichment with multiparameter flow cytometric and immunocytochemical analysis to detect, enumerate and characterize carcinoma cells in the blood. The assay can detect one epithelial cell or less in 1 ml of blood and has a greater sensitivety than conventional PCR or immunohistochemistry by 1–2 orders of magnitude. In addition, the assay facilitates the biological characterization and staging of carcinoma cells.

METHODS AND REAGENTS FOR THE RAPID AND EFFICIENT
ISOLATION OF CIRCULATING CANCER CELLS

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FIELD OF THE INVENTION

10   This invention relates to the fields of oncology
and diagnostic testing. The invention is useful for
cancer screening, staging, monitoring for chemotherapy
treatment responses, cancer recurrence or the like.
More specifically, the present invention provides

15   reagents, methods and test kits which facilitate
analysis and enumeration of tumor cells, or other rare
cells isolated from biological samples.

BACKGROUND OF THE INVENTION

20   Each year in the United States, approximately
600,000 new cases of cancer are diagnosed; one out of
every five people in this country will die from cancer
or from complications associated with its treatment.
Considerable efforts are continually directed at

25   improving treatment and diagnosis of this disease.
Most cancer patients are not killed by their
primary tumor. They succumb instead to metastases:
multiple widespread tumor colonies established by
malignant cells that detach themselves from the original

30   tumor and travel through the body, often to distant
sites. If a primary tumor is detected early enough, it
can often be eliminated by surgery, radiation, or
chemotherapy or some combination of those treatments.
Unfortunately, the metastatic colonies are harder to

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detect and eliminate and it is often impossible to treat all of them successfully. Therefore, from a clinical point of view, metastasis can be considered the conclusive event in the natural progression of cancer.

5 Moreover, the ability to metastasize is the property that uniquely characterizes a malignant tumor.

Cancer metastasis comprises a complex series of sequential events. These are: 1) extension from the primary locus into surrounding tissues; 2) penetration

10 into body cavities and vessels; 3) release of tumor cells for transport through the circulatory system to distant sites; 4) reinvasion of tissue at the site of arrest; and 5) adaptation to the new environment so as to promote tumor cell survival, vascularization and

15 tumor growth.

Based on the complexity of cancer and cancer metastasis and the frustration in treating cancer patients over the years, many attempts have been made to develop diagnostic tests to guide treatment and monitor

20 the effects of such treatment on metastasis or relapse. Such tests presumably could also be used for cancer screening, replacing relatively crude tests such as mammography for breast tumors or digital rectal exams for prostate cancers. Towards that goal, a number of

25 tests have been developed over the last 20 years and their benefits evaluated. One of the first attempts was the formulation of an immunoassay for carcinoembryonic antigen [CEA]. This antigen appears on fetal cells and reappears on tumor cells in certain cancers. Extensive

30 efforts have been made to evaluate the usefulness of testing for CEA as well as many other "tumor" antigens, such as PSA, CA 15.3, CA125, PSMA, CA27.29. These

efforts have proven to be somewhat futile as the appearance of such antigens in blood have not been generally predictive and are often detected when there is little hope for the patient. In the last few years,

5   however, one test has proven to be useful in the early detection of cancer, viz., Prostate Specific Antigen [PSA] for prostate cancers. When used with follow-up physical examination and biopsy, the PSA test has played a remarkable role in detecting prostate cancer early, at

10   the time when it is best treated.

Despite the success of PSA testing, the test leaves much to be desired. For example, high levels of PSA do not always correlate with cancer nor do they appear to be an indication of the metastatic potential of the

15   tumor. This may be due in part to the fact that PSA is a component of normal prostate tissue as well as other unknown factors. Moreover, it is becoming clear that a large percentage of prostate cancer patients will continue to have localized disease which is not life

20   threatening. Based on the desire to obtain better concordance between those patients with cancers that will metastasize and those that won't, attempts have been made to determine whether or not prostate cells are in the circulation. When added to high PSA levels and

25   biopsy data, the existence of circulating tumor cells might give indications as to how vigorously the patient should be treated.

The approach for determining the presence of circulating prostate tumor cells has been to test for

30   the expression of messenger RNA of PSA in blood. This is being done through the laborious procedure of isolating all of the mRNA from a blood sample and

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performing reverse transcriptase PCR. As of this date, (Gomella LG. J of Urology. 158:326-337(1997)) no good correlation exists between the presence of such cells in blood and the ability to predict which patients are in
5   need of vigorous treatment. It is noteworthy that PCR is difficult, if not impossible in many situations, to perform quantitatively, i.e., determine number of tumor cells per unit volume of biological sample. Additionally false positives are often observed using
10   this technique. There is an added drawback which is that there is a finite and practical limit to the sensitivity of this technique based on the sample size examined. Typically, the test is performed on 10^5 to 10^6 cells purified away from interfering red blood cells.
15   This corresponds to a practical lower limit of sensitivity of one tumor cell/ 0.1 ml of blood. Hence, there needs to be about 10 tumor cells in a ml of blood before signal is detectable. As a further consideration, tumor cells are often genetically
20   unstable. Accordingly, cancer cells having genetic rearrangements and sequence changes may be missed in a PCR assay as the requisite sequence complementarity between PCR primers and target sequences can be lost.
      In summary, a useful diagnostic test needs to be
25   very sensitive and reliably quantitative. If a blood test can be developed where the presence of a single tumor cell can be detected in one ml of blood, that would correspond on average to 3000 - 4000 total cells in circulation. In innoculum studies for establishing
30   tumors in animals, that number of cells can indeed lead to the establishment of a tumor. Further if 3000-4000 circulating cells represents 0.01% of the total cells in

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a tumor, then it would contain about 4×10^7 total cells.
A tumor containing that number of cells would not be
visible by any technique currently in existence. Hence,
if tumor cells are shed in the early stages of cancer, a

5   test with the sensitivity mentioned above would detect
the cancer. If tumor cells are shed in some functional
relationship with tumor size, then a quantitative test
would be beneficial to assessing tumor burden.
Heretofore there has been no information regarding the

10   existence of circulating tumor cells in very early
cancers. Further, there are very considerable doubts in
the medical literature regarding the existence of such
cells and the potential of such information. The
general view is that tumors are initially well confined

15   and hence there will be few if any circulating cells in
early stages of disease. Also, there are doubts that the
ability to detect cancer cells early on will give any
useful information.

Based on the above, it is apparent that a method

20   for identifying those cells in circulation with
metastatic potential prior to establishment of a
secondary tumor is highly desirable, particularly early
on in the cancer. To appreciate the advantage such a
test would have over conventional immunoassays, consider

25   that a highly sensitive immunoassay has a lower limit of
functional sensitivity of 10^{-17} moles. If one tumor cell
can be captured from a ml of blood and analyzed, the
number of moles of surface receptor, assuming 100,000
receptors per cell would be 10^{-19} moles. Since about 300

30   molecules can be detected on a cell such an assay would
have a functional sensitivity on the order of 10^{-22}
moles, which is quite remarkable. To achieve that level

of sensitivity in the isolation of such rare cells, and
to isolate them in a fashion which does not compromise
or interfere with their characterization is a formidable
task.

5   Many laboratory and clinical procedures employ bio-
specific affinity reactions for isolating rare cells
from biological samples. Such reactions are commonly
employed in diagnostic testing, or for the separation of
a wide range of target substances, especially biological

10   entities such as cells, proteins, bacteria, viruses,
nucleic acid sequences, and the like.

Various methods are available for analyzing or
separating the above-mentioned target substances based
upon complex formation between the substance of interest

15   and another substance to which the target substance
specifically binds. Separation of complexes from
unbound material may be accomplished gravitationally,
e.g. by settling, or, alternatively, by centrifugation
of finely divided particles or beads coupled to the

20   target substance. If desired, such particles or beads
may be made magnetic to facilitate the bound/free
separation step. Magnetic particles are well known in
the art, as is their use in immune and other bio-
specific affinity reactions. See, for example, US

25   Patent No. 4,554,088 and Immunoassays for Clinical
Chemistry, pp. 147-162, Hunter et al. eds., Churchill
Livingston, Edinburgh (1983). Generally, any material
which facilitates magnetic or gravitational separation
may be employed for this purpose. However, it has

30   become clear that magnetic separation means are the
method of choice.

Magnetic particles can be classified on the basis

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of size as large (1.5 to about 50 microns), small (0.7-
1.5 microns), or colloidal (<200nm), which are also
referred to as nanoparticles. The latter, which are
also known as ferrofluids or ferrofluid-like materials

5   and have many of the properties of classical
ferrofluids, are sometimes referred to herein as
colloidal, superparamagnetic particles.

Small magnetic particles of the type described
above are quite useful in analyses involving bio-

10   specific affinity reactions, as they are conveniently
coated with biofunctional polymers (e.g., proteins),
provide very high surface areas and give reasonable
reaction kinetics. Magnetic particles ranging from 0.7-
1.5 microns have been described in the patent

15   literature, including, by way of example, US Patent Nos.
3,970,518; 4,018,886; 4,230,685; 4,267,234; 4,452,773;
4,554,088; and 4,659,678. Certain of these particles
are disclosed to be useful solid supports for
immunological reagents.

20   Small magnetic particles, such as those mentioned
above, generally fall into two broad categories. The
first category includes particles that are permanently
magnetizable, or ferromagnetic; and the second comprises
particles that exhibit bulk magnetic behavior only when

25   subjected to a magnetic field. The latter are referred
to as magnetically responsive particles. Materials
displaying magnetically responsive behavior are
sometimes described as superparamagnetic. However,
materials normally considered ferromagnetic, e.g.,

30   magnetic iron oxide, may be characterized as
superparamagnetic when provided in crystals of about
30nm or less in diameter. Relatively larger crystals of

ferromagnetic materials, by contrast, retain permanent
magnet characteristics after exposure to a magnetic
field and tend to aggregate thereafter due to strong
particle-particle interactions.

5   Like the small magnetic particles mentioned above,
large magnetic particles (> 1.5 microns to about 50
microns) can also exhibit superparamagnetic behavior.
Typical of such materials are those described by
Ugelstad in US Patent No.4,654267 and manufactured by

10   Dynal, (Oslo, Norway). The Ugelstad process involves
the synthesis of polymer particles which are caused to
swell and magnetite crystals are embedded in the swelled
particles. Other materials in the same size range are
prepared by synthesizing the polymer particle in the

15   presence of dispersed magnetite crystals. This results
in the trapping of magnetite crystals in a polymer
matrix, thus making the resultant materials magnetic.
In both cases, the resultant particles have
superparamagnetic behavior, which is manifested by the

20   ability to disperse readily upon removal of the magnetic
field. Unlike magnetic colloids or nanoparticles
previously referred to and discussed in further detail
below, these materials, as well as small magnetic
particles, are readily separated with simple laboratory

25   magnetics because of the mass of magnetic material per
particle. Thus, separations are effected in gradients
from as low as a few hundred gauss/cm on up to about 1.5
kilogauss/cm. Colloidal magnetic particles, (below
approximately 200nm),on the other hand, require

30   substantially higher magnetic gradients because of their
diffusion energy, small magnetic mass per particle and
Stokes drag.

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US Patent No. 4,795,698 to Owen et al. relates to
polymer-coated, colloidal, superparamagnetic particles
which are produced by the formation of magnetite from
Fe^{+2}/Fe^{+3} salts in the presence of polymer. US Pat. No.
5   4,452,773 to Molday describes a material similar in
properties to those described in Owen et al., which is
produced by forming magnetite and other iron oxides from
Fe^{+2}/Fe^{+3} via base addition in the presence of very high
concentrations of dextran. The resulting particles from
10   both procedures exhibit an appreciable tendency not to
settle from aqueous suspensions for observation periods
as long as several months. Materials so produced have
colloidal properties and have proved to be very useful
in cell separation. The Molday technology has been
15   commercialized by Miltenyi Biotec, Bergisch Gladbach,
Germany and Terry Thomas, Vancouver, Canada.

Another method for producing superparamagnetic,
colloidal particles is described in US Pat. No.
5,597,531. In contrast to the particles described in
20   the Owen et al., or Molday patents, these latter
particles are produced by directly coating a
biofunctional polymer onto pre-formed superparamagnetic
crystals which have been dispersed by high power sonic
energy into quasi-stable crystalline clusters ranging
25   from 25 to 120nm. The resulting particles, referred to
herein as direct-coated particles, exhibit a
significantly larger magnetic moment than colloidal
particles of the same overall size, such as those
described by Molday or Owen et al.

30   Magnetic separation techniques are known wherein a
magnetic field is applied to a fluid medium in order to
separate ferromagnetic bodies from the fluid medium. In

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contrast, the tendency of colloidal, superparamagnetic
particles to remain in suspension, in conjunction with
their relatively weak magnetic responsiveness, requires
the use of high-gradient magnetic separation (HGMS)

5   techniques in order to separate such particles from a
non-magnetic fluid medium in which they are suspended.
In HGMS systems, the gradient of the magnetic field,
i.e., the spatial derivative, exerts a greater influence
upon the behavior of the suspended particles than is

10   exerted by the strength of the field at a given point.

HGMS systems can be divided into two broad
categories. One such category includes magnetic
separation systems which employ a magnetic circuit that
is entirely situated externally to a separation chamber

15   or vessel. Examples of such external separators are
described in US Pat. No. 5,186,827 to Liberti et al. In
several of the embodiments described in this patent, the
requisite magnetic field gradient is produced by
positioning permanent magnets around the periphery of a

20   non-magnetic container such that the like poles of the
magnets are in a field-opposing configuration. The
extent of the magnetic field gradient within the test
medium that may be obtained in such a system is limited
by the strength of the magnets and the separation

25   distance between the magnets. Hence, there is a finite
limit to gradients that can be obtained with external
gradient systems.

Another type of HGMS separator utilizes a
ferromagnetic collection structure that is disposed

30   within the test medium in order to 1) intensify an
applied magnetic field and 2) produce a magnetic field
gradient within the test medium. In one known type of

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internal HGMS system, fine steel wool or gauze is packed
within a column that is situated adjacent to a magnet.
The applied magnetic field is concentrated in the
vicinity of the steel wires so that suspended magnetic
5   particles will be attracted toward, and adhere to, the
surfaces of the wires. The gradient produced on such
wires is inversely proportional to the wire diameter,
such that magnetic reach decreases with increasing
diameter. Hence, very high gradients can be generated.
10   One drawback of internal gradient systems is that
the use of steel wool, gauze material, or steel
microbeads, may entrap non-magnetic components of the
test medium by capillary action in the vicinity of
intersecting wires or within interstices between
15   intersecting wires. Various coating procedures have
been applied to such internal gradient columns (see,
e.g., US Patent Nos. 5,693,539 to Miltenyi and 4,375,407
to Kronick), however, the large surface area in such
systems still creates recovery concerns due to
20   adsorption. Hence, internal gradient systems are not
desirable, particularly when recovery of very low
frequency captured entities is the goal of the
separation. Furthermore, they make automation difficult
and costly. Both the materials described by Owen et
25   al., and Molday require the use of such high gradient
columns.

In contrast, HGMS approaches using external
gradients for cell separation provide a number of
conveniences. Firstly, simple laboratory containers
30   such as test tubes, centrifuge tubes or even vacutainers
(used for blood collection) can be employed. When
external gradients are of the kind that produce

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monolayers of separated cells, as is the case with
quadrupole/hexapole devices of the above-mentioned US
Pat. No.5,186,827 or the opposing dipole arrangement
described in US Patent 5,466,574 to Liberti et al.,

5   washing of cells or subsequent manipulations are
facilitated. Further, recoveries of cells from tubes or
similar containers is a simple and efficient process.
This is particularly the case when compared to
recoveries from high gradient columns. Such separation

10   vessels also provide another important feature, which is
the ability to reduce sample volume. For example, if a
particular human blood cell subset, (e.g. magnetically
labeled CD 34⁺ cells), is isolated from a 10 ml blood
sample diluted 50% with buffer to reduce viscosity, a 15

15   ml conical test tube may be employed as the separation
vessel in an appropriate quadrupole magnetic device.
Starting with 15 mls of solution, a first separation is
performed, and the recovered cells are resuspended in 3
mls. A second wash/separation is then performed and the

20   isolated cells resuspended in a final volume of 200 ul.
After the washes and/or separations and resuspensions
to remove non-bound cells, CD 34⁺ cells can effectively
be resuspended in a volume of 200 μl. When done
carefully in appropriately treated vessels using direct-

25   coated ferrofluids which have been optimized for these
separators, cell recovery is quite efficient in the 40-
90% range depending on antigen density. Such techniques
and reagents are essential to achieve the degree of
sensitivity required for the kinds of cancer testing

30   mentioned above.
The efficiency with which magnetic separations can
be done and the recovery and purity of magnetically

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labeled cells will depend on many factors. These
include such considerations as the number of cells being
separated, the receptor density of such cells, the
magnetic load per cell, the non-specific binding (NSB)

5   of the magnetic material, the technique employed, the
nature of the vessel, the nature of the vessel surface,
the viscosity of the medium and the magnetic separation
device employed. If the level of non-specific binding
of a system is substantially constant, as is usually the

10   case, then as the target population decreases so will
the purity. As an example, a system with 0.8 % NSB that
recovers 80% of a population which is at 0.25% in the
original mixture will have a purity of 25%. Whereas, if
the initial population was at 0.01% (one target cell in

15   10^6 bystander cells), and if the NSB were 0.001%, then
the purity would be 8%. The greater the purity, the
easier and better the analysis. Hence, it is clear that
extremely low non specific binding is required to
perform meaningful rare cell analysis.

20   Less obvious is the fact that the smaller the
population of a targeted cell, the more difficult it
will be to magnetically label and to recover.
Furthermore, labeling and recovery will markedly depend
on the nature of magnetic particle employed. For

25   example, when cells are incubated with large magnetic
particles, such as Dynal beads, cells are labeled
through collisions created by mixing of the system, as
the beads are too large to diffuse effectively. Thus,
if a cell were present in a population at a frequency of

30   1 cell per ml of blood or even less, as may be the case
for tumor cells in very early cancers, then the
probability of labeling target cells will be related to

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the number of magnetic particles added to the system and
the length of time of mixing. Since mixing of cells
with such particles for substantial periods of time
would be deleterious, it becomes necessary to increase

5   particle concentration as much a possible. There is,
however, a limit to the quantity of magnetic particle
that can be added, as one can substitute a rare cell
mixed in with other blood cells for a rare cell mixed in
with large quantities of magnetic particles upon

10   separation. The latter condition does not markedly
improve the ability to enumerate the cells of interest
or to examine them.

There is another drawback to the use of large
particles to isolate cells in rare frequencies (1 to 50

15   cells per ml of blood). Despite the fact that large
magnetic particles allow the use of external gradients
of very simple design and relatively low magnetic
gradient, large particles tend to cluster around cells
in a cage-like fashion making the cells difficult to see

20   or to analyze. Hence, the magnetic particles must be
released from the target cells before analysis, and
releasing the particles clearly introduces other
complications.

Based on the foregoing, high gradient magnetic

25   separation with an external field device employing
highly magnetic, low non-specific binding, colloidal
magnetic particles is the method of choice for
separating a cell subset of interest from a mixed
population of eukaryotic cells, particularly if the

30   subset of interest comprises but a small fraction of the
entire population. Such materials, because of their
diffusive properties, readily find and magnetically

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label rare events, such as tumor cells in blood. Such
separation generally relies upon the identification of
cell surface antigens that are unique to a specific cell
subset of interest, which in the case of tumor cells,

5   can be tumor antigens to which appropriate monoclonal
antibody conjugated ferrofluids can be targeted.
Alternatively, when examining a blood sample,
determinants on classes of cells such as epithelial
cells, which are normally not found in blood, can

10   provide an appropriate receptor.

There are other good reasons to employ a colloidal
magnetic material for such separations, providing an
appropriate magnetic loading can be achieved. With
appropriate loading, a sufficient force is exerted on a

15   cell such that isolation can be achieved even in a media
as viscous as that of moderately diluted whole blood.
As noted, colloidal magnetic materials below about 200
nanometers will exhibit Brownian motion which markedly
enhances their ability to collide with and magnetically

20   label rare cells. This is demonstrated in US Patent No.
5,541,072 where results of very efficient tumor cell
purging experiments are described employing colloidal
magnetic particles or ferrofluids having a mean diameter
of 100 nm. Just as importantly, colloidal materials

25   having a particle size at or below this size range do
not generally interfere with examination of cells.
Cells so retrieved can be examined by flow cytometry,
laser scanning microscopy, or by microscopy employing
visible or fluorescent techniques.

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SUMMARY OF THE INVENTION

The present invention is based on several important discoveries which have significant clinical ramifications for the diagnosis and treatment of cancer.

5   These are 1) tumor cells are present in the blood of patients considered to have clinically localized, primary tumors; 2) the number of tumor cells present in the circulation is correlatable with all stages of cancer from its inception to its terminal stages; and 3)

10   changes in the number of tumor cells present in the circulation is indicative of disease progression. A decrease in the numbers of circulating tumor cells is indicative of improvement in patient status or efficacy of treatment, whereas an increase indicates a worsening

15   of the disease.

The present invention provides a rapid and efficient screening method for the characterization of not only tumor cells, but also rare cells, or other biological entities from biological samples. The method

20   of the invention provides highly sensitive analytical techniques which enable efficient enrichment for entities of interest. This two stage methodology which ensures enrichment of target bioentities while eliminating a substantial amount of debris and other

25   interfering substances prior to analysis, allows for examination of sample sizes which would otherwise be impractical. The method described herein combines elements of immunomagnetic enrichment with multiparameter flow cytometric, microscopic and

30   immunocytochemical analysis in a unique way. Other means of enrichment such as density gradient centrifugation or panning or alteration of target cell

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density by appropriate labeling may also be utilized. According to a preferred embodiment, the method of the invention enables assaying whole blood for cancer staging, monitoring and screening. The sensitive nature

5 of the assay facilitates the detection of residual disease, thus making it possible to monitor for cancer recurrence.

In one embodiment of the invention, a biological specimen, which comprises a mixed cell population

10 suspected of containing the rare cell of interest is obtained from a patient. An immunomagnetic sample is then prepared by mixing the biological specimen with i. magnetic particles which are coupled to a biospecific ligand specifically reactive with a rare cell

15 determinant or a class of determinants different than those found on blood cells, to the substantial exclusion of other sample components, and ii. at least one biospecific reagent which labels rare cells. The resulting immunomagnetic sample is subjected to a

20 magnetic field which is effective to separate the sample into an unlabeled fraction and a labeled, magnetic fraction including the rare cell of interest, if any is present in the specimen. The cell population so isolated is then analyzed to determine the presence and

25 number of rare cells. In a preferred embodiment the particles used in this method are colloidal nanoparticles.

In another embodiment of the invention, a biological specimen is obtained from a patient. An

30 immunomagnetic sample is then prepared wherein the biological specimen is mixed with colloidal magnetic particles which have been coupled to a monoclonal

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antibody reactive with the rare cell determinant or a

class of determinants different than those found on

blood cells. As an alternative to monoclonal

antibodies, single chain or engineered fragments of

5   antibodies may be employed. The preparation is subjected

to a magnetic field, enriching the rare cell component

of the specimen. A second set of monoclonal antibodies,

labeled with reporter molecules, are added to the sample

and the cells are again magnetically separated in order

10   to remove unbound reagent to lower background staining.

A nucleic acid dye or other reporter molecule capable of

identifying objects as cells, also referred to herein as

a cell specific dye, is added to the sample to allow

exclusion of any residual non-nucleated cells or other

15   sample components prior to analysis by flowcytometry,

microscopy, or other analytical platforms. Cell

specific dyes may be reactive with DNA, RNA, protein, or

lipids such that the amount of signal obtained is

typical for that obtained for cells or the image

20   obtained reveals typical features of a cell, such as

cell and nuclear membranes, nucleus, and mitochondria.

         In a further embodiment of the invention, the

isolated cells are subjected to immunocytochemical

analysis by flowcytometry or other analytical platforms.

25   Such analysis facilitates diagnosis and provides

important information to the clinician.

         The method of the invention may be used to

assess residual cancer cells in circulation following

medical, radiation, or surgical treatment to eradicate

30   the tumor. The method may be also be performed

periodically over a course of years to assess the

patient for the presence and number of tumor cells in

the circulation as an indicator of occurrence, recurrence and/or progression of disease.

In yet another aspect of the present invention, a coated, magnetic particle is provided which comprises a nanoparticle core of magnetic material, and a base coating material on the magnetic core in an amount sufficient to hinder non-specific binding of biological macromolecules to the magnetic core. These magnetic particles are characterized by extremely low non-specific binding as well as highly efficient target capture which are essential to achieve a level of enrichment the enrichment required to effectively isolate very rare cells. In an alternative embodiment, a coated, magnetic particle is provided which comprises the following: i. a nanoparticle core of magnetic material; ii. a base coating material that forms a discontinous coating on the magnetic core, providing at least one area of discontinuity which, if accessible, contributes to non-specific binding of the base coated particle to biological macromolecules; and iii. an additional coating material that hinders access to the areas of discontinuity by biological macromolecules. The magnetic core material of the particles described immediately above may comprise at least one transition metal oxide and a suitable base coating material comprises a protein. Proteins suitable for coating magnetic particles include but are not limited to bovine serum albumin and casein. The additional coating material may be the original coating proteins or one member of a specific binding pair which is coupled to the base material on the magnetic core. Exemplary specific binding pairs include biotin-streptavidin,

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antigen-antibody, receptor-hormone, receptor-ligand, agonist-antagonist, lectin-carbohydrate, Protein A-antibody Fc, and avidin-biotin. In one embodiment, the member of the specific binding pair is coupled to the base coating material through a bifunctional linking compound. Exemplary biofunctional linking compounds include succinimidyl-propiono-dithiopyridine (SPDP), and sulfosuccinimidil-4-[maleimidomethyl]cyclohexane-1-carboxylate (SMCC), however a variety of other such heterobifunctional linker compounds are available from Pierce, Rockford, Ill.

The coated magnetic particles of the invention preferably have between 70-90% magnetic mass. In a preferred embodiment, a major portion of the magnetic particles have a particle size in the range of 90-150 nm. Particles may be synthesized such that they are more monodisperse, e.g., in the range of 90-120 nm or 120-150 nm. The particles of the invention are typically suspended in a biologically compatible medium.

In a further aspect of the present invention, a test kit is provided for screening a patient sample for the presence of circulating rare cells. The screening kit comprises:

i. coated, magnetic nanoparticles coupled, directly or indirectly, to a biospecific ligand that has affinity for a first characteristic determinant on a rare cell; ii. at least one biospecific reagent having binding specificity for a second characteristic determinant present on a rare cell; and iii. a cell specific dye for excluding other non-target or sample entities from analysis.

In a particularly preferred embodiment, a kit is

provided for screening biological samples for
circulating cancer cells. The screening kit comprises:
i. coated, magnetic nanoparticles coupled, directly or
indirectly to a biospecific ligand that has affinity for
5   a first characteristic determinant on a cancer cell;
ii. at least one biospecific reagent having binding
specificity for a second characteristic determinant
present on a cancer cell; and iii. a cell specific dye
for excluding non-target entities from analysis. The
10   kits provided herein may further include an antibody
which has affinity for non-rare, or non-tumor cells, a
biological buffer, a permabilization buffer, a protocol
and, if desired, an information sheet. In a preferred
embodiment, the colloidal magnetic particles are
15   conjugated to anti-EpCAM (an antibody having binding
specificity for epithelial cell adhesion molecule), the
biospecific reagents comprise a panel of monoclonal
antibodies and the cell specific dye stains nucleic
acids.

20   The kits of the invention may contain reagents for
diagnosing the type of the metastatic cancer cells in
the circulation as well as the metastatic potential and
aggressiveness of such cells. In this embodiment the
kit contains the reagents recited above, yet also
25   comprises additional antibody markers to facilitate
cancer diagnosis. Using breast cancer as an example,
such antibodies may include anti-MUC-1, anti-estrogen
receptor, anti-progesterone receptor, anti-CA27.29,
anti-CA15.5, anti-cathepsin D, anti-p53, anti- urokinase
30   type plasminogen activator, anti-epidermal growth
factor, anti-epidermal growth factor receptor, anti-
BRCA1, anti-BRCA2, anti-prostate specific antigen, anti-

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plasminogen activator inhibitor and/or anti-Her2-neu
antibodies. Additional markers for aggressiveness and
invasiveness are Lewis a (Lea), sialyl Lewis a (sLea),
the intergrins (CD49b, CD49c, CD29), gelatinase A and B

5   (MMP-2, MMP-9), tissue collagenase (MMP-1),fibroblast
activation protein (FAP), guanidinobenzoatase, CEA, S100
family (S100A4, mtsl, 18A2/mtsl, pEL-98, p9Ka,
metastasin), the Cyclins A and E, p27, p53, vascular
endothelilal growth factor (VGEF) and E-Cadherin.

10   In yet another embodiment of the invention, a test
kit is provided for monitoring a patient for recurrence
of the cancer, and/or response to therapy. This
particular kit may also be used to assess high risk
patients for the presence of particular tumor cells in

15   the blood. A kit suitable for monitoring a patient
would include containers, colloidal magnetic particles
conjugated to anti-EpCAM, at least one monoclonal
antibody specific for the particular cancer cells for
which the patient is being monitored and a fluorescent

20   reporter molecule which can identify the objects as
cells, such as nucleic acid or membrane dyes. A kit
suitable for monitoring breast cancer patients comprises
an antibody having binding affinity for a particular
breast cancer marker, for example Her-2-neu. The kits

25   described above are suitable for screening, diagnosing
and monitoring patients for breast cancer. It will be
appreciated by those skilled in the art that many
different cancers may be screened, diagnosed and
monitored according to the present invention simply by

30   varying the antibodies provided in the test kit. For
example, if a test subject were being assessed for the
presence of prostate cancer, antibodies specific for

prostate specific antigen may be employed. Other
markers for prostate cancer include prostatic acid
phosphatase, creatine kinase, thymosin b-15, p53, HPC1
basic prostate gene, and prostate specific membrane

5 antigen.

There is general agreement that the hallmark of
successful treatment of cancer is early diagnosis.
Based on the data presented herein, it appears that the
blood test of the present invention may be used to

10 screen the blood of patients who do not have a diagnosis
of cancer, in order to detect cancer earlier than is
possbile using other existing methods. Such patients
may include those with a family history of certain
cancers, patients with certain mutations known to be

15 associated with cancer, etc.

Since cancer cells invade surrounding tissue and
breakdown tissue barriers, we hypothesize that tumor
cells enter the tissue space and capillaries to
eventually end up in the blood very early in the

20 development of a solid tumor, i.e., when the tumor
contains 10^4-10^6 tumor cells. See Figure 8. At that
point in time, the tumor cells undergo apoptotic cell
death, or become dormant because they are not yet able
to survive or grow, respectively, in an ectopic

25 environment. There are no techniques at present to
detect such small primary tumors. There are sensitive
techniques that are available for detecting certain
types of cancer when the tumors are larger. For
example, mammography can detect 2×10^8 breast cancer

30 cells at best. More often, tumors of the breast are
detected when there are between 5×10^8 to 10^9 tumor
cells. At this early stage, we hypothesize that most

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shed tumor cells will die. However, during the many generations that occur as a tumor grows between 10^6 to 10^{8-9} tumor cells, the genetically unstable clone of tumor cells undergoes further genetic changes giving

5   rise to more rapidly growing and aggressive mutant cells. It is very likely these cells that go on to establish secondary tumors. However, in the majority of tumors, the diagnosis is made very late, e.g., pancreas, stomach, ovary, kidney, lung, colon, etc., are usually

10   diagnosed when there are 10^{10}-10^{12} tumor cells. By this time the tumor has frequently invaded surrounding tissues and/or has metastasized. In light of the foregoing, it is clear that any test which would effectively detect circulating cancer cells prior to the

15   establishment of a secondary tumor would be extremely beneficial in the diagnosis and treatment of cancer. The blood test described herein enables such detection.

BRIEF DESCRIPTION OF THE DRAWINGS

20   **Figure 1** shows the results of model experiments in which known number of tumor cells are spiked into peripheral blood and retrieved after immunomagnetic selection and analysis by either microscopy (Panel A) or flowcytometry (Panel B).

25

     Figure 2 shows flowcytometric analysis of cell suspensions obtained after immunomagnetic cell selection from 10 ml of blood from a patient having distant metastasis of carcinoma of the breast, drawn 48, 175 and

30   300 days after this patient entered the study. After immunomagnetic selection, the cells were stained with an epithelial cell specific phycoerythrin (PE) conjugated

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monoclonal antibody, a leukocyte specific CD45 PerCP conjugated monoclonal antibody and a nucleic acid dye. Events passing a threshold on the nucleic acid dye were acquired into listmode and 85% of the sample was

5   analyzed. The tumor cells are highlighted and illustrated in black and their number is shown in the top right corner; the background events, consisting of residual leukocytes and debris, are illustrated in gray.

10   **Figure** 3 shows epithelial cell number in 10 ml of blood and clinical activity of the disease at different time points for eight patients with active carcinoma of the breast. The clinical activity of the disease was classified in categories 1 through 4, as set out in

15   Table 1. The bars at the top represent the length of time of chemotherapy. Panel A, adriamycine (ADR) 90 and 110 mg/m^2 respectively, Panel B, ADR 30 mg/m^2/week, Vinorelbine (Vin) 20 mg/m^2/week, ADR 160 mg, ADR 20 mg/m^2/week, Panel C, vincristine (Vinc) 0.7 mg/m^2/week,

20   metotrexate (MTX) 30 mg/m^2/week, Panel D, vinblastine (Vinb) 7 mg/m^2/week, ADR 20 mg/m^2/week, Vinb 6 mg/m^2/week, 5-fluoruracil (5FU) 700 mg/m^2/week. Panel E, Vin 20 mg/m^2/week; 5FU 800 mg/m^2/week + Leukovorin 50 mg/m^2/week. Panel F, ifosfamide (IF) 18 mg/m^2/week; 5FU

25   850 mg/m^2/week + Leukovorin 35 mg/m^2/week, 5FU 605 mg/m^2/week; Vin 20 mg/m^2/week+ Leukovorin 30 mg/m^2/week. Panel G, Vin 20 mg/m^2/week, Panel H, Vin 20 mg/m^2/week

        Figures 4A-4D are a series of micrographs showing
30   the results obtained following analysis of immunomagnetically selected cells from peripheral blood of patients with a history of breast carcinoma. Panel

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A, cells from a patient three years after surgery
(T2N1M0) staining positive for cytokeratin. Panel B,
cell from a patient eight years after surgery (T2N1M1)
in complete remission stained with Wright Giemsa. Panel

5   C and D cells from a patient 2 years after surgery
(T2N0M0) stained with Wright Giemsa. The images were
taken with a Pixera digital camera with a 100X
objective.

10   **Figures 5A-5C** are a series of graphs showing the
correlation between severity of disease and circulating
epithelial cell number in three patients with prostate
cancer.

15   **Figure 6** is a graph which shows that circulating
epithelial cell number in patients with colon cancer is
significantly decreased after surgical removal of the
tumor.

20   **Figure 7** is a graph which shows that circulating
epithelial cell number in patients with metastatic
disease of the colon increases with the severity and
extent of metastatic disease.

25   **Figure 8** is a schematic diagram showing the
progression of cancer from a primary tumor to growing
metastases.

DETAILED DESCRIPTION OF THE INVENTION

30   According to a preferred embodiment, the present
invention provides compositions, methods and kits for
the rapid and efficient isolation of rare target

bioentities from biological samples. The methods
described may be used effectively to isolate and
characterize tumor cells present in a blood sample while
at the same time minimizing the selection of non-
specifically bound cells.

Many clinicians believe that cancer is an organ-
confined disease in its early stages. Based on the data
presented herein, it appears that this notion is
incorrect. Indeed, the data reveal that cancer is often
a systemic disease by the time it is first detected
using methods currently available. Hence, the presence
of tumor cells in the circulation can be used to screen
for cancer in place of, or in conjunction with, other
tests, such as mammography, or measurements of PSA. By
employing appropriate mononclonal antibodies directed to
specific markers on or in cells, or by using other
assays for cell protein expression, or by the analysis
of cellular mRNA, the organ origin of such cells may
readily be determined, e.g., breast, prostate, colon,
lung, ovarian or other non-hematopoietic cancers. Thus,
in cases where cancer cells can be detected, while there
are essentially no clinical signs of a tumor, it will be
possible to identify their presence as well as the organ
of origin. Because screening can be done with the
relatively simple blood test of the present invention
described herein, which functions with a high degree of
sensitivity, the test can be thought of as a "whole body
biopsy". Furthermore, based on the data set forth
herein, cancer should be thought of as a blood borne
disease characterized by the presence of potentially
very harmful metastatic cells, and therefore, treated
accordingly. In cases where there is absolutely no

detectable evidence of circulating tumor cells, e.g.,
following surgery, it may be possible to determine from
further clinical study whether follow-up treatment, such
as radiation or chemotherapy is required. Determining
5   the need not to treat, given the costs of such
therapies, is a significant and beneficial piece of
clinical information.

It is also clear from the present data that the
number of tumor cells in the ciruclation is related to
10   the stage of progression of the disease, from its
inception to the final phases of disease.

The term "target bioentities" as used herein refers
to a wide variety of materials of biological or medical
interest. Examples include hormones, proteins,
15   peptides, lectins, oligonucleotides, drugs, chemical
substances, nucleic acid molecules, (e.g., RNA and/or
DNA) and particulate analytes of biological origin,
which include bioparticles such as cells, viruses,
bacteria and the like. In a preferred embodiment of the
20   invention, rare cells, such as fetal cells in maternal
circulation, or circulating cancer cells may be
efficiently isolated from non-target cells and/or other
bioentities, using the compositions, methods and kits of
the present invention. The term "biological specimen"
25   includes, without limitation, cell-containing bodily,
fluids, peripheral blood, tissue homogenates, nipple
aspirates, and any other source of rare cells that is
obtainable from a human subject. An exemplary tissue
homogenate may be obtained from the sentinel node in a
30   breast cancer patient. The term "determinant", when
used in reference to any of the foregoing target
bioentities, may be specifically bound by a biospecific

ligand or a biospecific reagent, and refers to that
portion of the target bioentity involved in, and
responsible for, selective binding to a specific binding
substance, the presence of which is required for

5   selective binding to occur. In fundamental terms,
determinants are molecular contact regions on target
bioentities that are recognized by receptors in specific
binding pair reactions. The term "specific binding
pair" as used herein includes antigen-antibody,

10   receptor-hormone, receptor-ligand, agonist-antagonist,
lectin-carbohydrate, nucleic acid (RNA or DNA)
hybridizing sequences, Fc receptor or mouse IgG-protein
A, avidin-biotin, streptavidin-biotin and virus-receptor
interactions. Various other determinant-specific

15   binding substance combinations are contemplated for use
in practicing the methods of this invention, such as
will be apparent to those skilled in the art. The term
"antibody" as used herein, includes immunoglobulins,
monoclonal or polyclonal antibodies, immunoreactive

20   immunoglobulin fragments, and single chain antibodies.
Also contemplated for use in the invention are peptides,
oligonucleotides or a combination thereof which
specifically recognize determinants with specificity
similar to traditionally generated antibodies. The term

25   "detectably label" is used to herein to refer to any
substance whose detection or measurement, either
directly or indirectly, by physical or chemical means,
is indicative of the presence of the target bioentity in
the test sample. Representative examples of useful

30   detectable labels, include, but are not limited to the
following: molecules or ions directly or indirectly
detectable based on light absorbance, fluorescence,

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reflectance, light scatter, phosphorescence, or
luminescence properties; molecules or ions detectable by
their radioactive properties; molecules or ions
detectable by their nuclear magnetic resonance or

5   paramagnetic properties. Included among the group of
molecules indirectly detectable based on light
absorbance or fluorescence, for example, are various
enzymes which cause appropriate substrates to convert,
e.g., from non-light absorbing to light absorbing

10   molecules, or from non-fluorescent to fluorescent
molecules. The phrase "to the substantial exclusion of"
refers to the specificity of the binding reaction
between the biospecific ligand or biospecific reagent
and its corresponding target determinant. Biospecific

15   ligands and reagents have specific binding activity for
their target determinant yet may also exhibit a low
level of non-specific binding to other sample
components. The term "early stage cancer" as used
herein refers to those cancers which have been

20   clinically determined to be organ-confined. Also
included are tumors too small to be detected by
conventional methods such as mammography for breast
cancer patients, or X-rays for lung cancer patients.
While mammography can detect tumors having approximately

25   2×10^8 cells, the methods of the present invention
should enable detection of circulating cancer cells from
tumors approximating this size or smaller. The term
"enrichment" as used herein refers to the enrichment of
mononuclear cells from a biological sample. In cases

30   where peripheral blood is used as the starting
materials, red cells are not counted when assessing the
extent of enrichment. Using the method of the present

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invention, circulating epithelial cells may be enriched
relative to leucocytes to the extent of at least 2,500
fold, more preferably 5,000 fold and most preferably
10,000 fold.   The preferred magnetic particles for use
5   in carrying out this invention are particles that behave
as colloids. Such particles are characterized by their
sub-micron particle size, which is generally less than
about 200 nanometers (nm) (0.20 microns), and their
stability to gravitational separation from solution for
10   extended periods of time. In addition to the many other
advantages, this size range makes them essentially
invisible to analytical techniques commonly applied to
cell analysis. Particles within the range of 90-150 nm
and having between 70-90% magnetic mass are contemplated
15   for use in the present invention. Suitable magnetic
particles are composed of a crystalline core of
superparamagnetic material surrounded by molecules which
are bonded, e.g., physically absorbed or covalently
attached, to the magnetic core and which confer
20   stabilizing colloidal properties. The coating material
should preferably be applied in an amount effective to
prevent non specific interactions between biological
macromolecules found in the sample and the magnetic
cores. Such biological macromolecules may include
25   sialic acid residues on the surface of non-target cells,
lectins, glyproteins and other membrane components. In
addition, the material should contain as much magnetic
mass/nanoparticle as possible. The size of the magnetic
crystals comprising the core is sufficiently small that
30   they do not contain a complete magnetic domain. The
size of the nanoparticles is sufficiently small such
that their Brownian energy exceeds their magnetic

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moment. As a consequence, North Pole, South Pole
alignment and subsequent mutual attraction/repulsion of
these colloidal magnetic particles does not appear to
occur even in moderately strong magnetic fields,

5   contributing to their solution stability. Finally, the
magnetic particles should be separable in high magnetic
gradient external field separators. That characteristic
facilitates sample handling and provides economic
advantages over the more complicated internal gradient

10   columns loaded with ferromagnetic beads or steel wool.
Magnetic particles having the above-described properties
can be prepared by modification of base materials
described in U.S. Patents Nos. 4,795,698, 5,597,531 and
5,698,271. Their preparation from those base materials

15   is described below.

        Malignant tumors are characterized by their ability
to invade adjacent tissue. In general, tumors with a
diameter of 1 mm are vascularized and animal studies
show that as much as 4% of the cells present in the

20   tumor can be shed into the circulation in a 24 hour
period (Butler, TP & Gullino PM, 1975 Cancer Research
35:512-516). The shedding capacity of a tumor is most
likely dependent on the aggressiveness of the tumor.
Although tumor cells are shed into the circulation on a

25   continous basis, it is believed that none or only a
small fraction will give rise to distant metastasis
(Butler & Gullino, *supra*). Using the following
assumptions, one can approximate the frequency of tumor
cells in circulation as follows: 1. A tumor with a

30   diameter of 1 mm contains 10^7 cells, and 4% or 4×10^5
cells will be shed into the circulation in a 24 hour
period; 2. tumor cells only survive one circulatory

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cycle; 3. a blood volume of about 5 liters; and 4. a
cardiac output of 5000 ml / minute. In such a case, the
frequency of tumor cells in peripheral blood of a
patient with a 1-mm diameter tumor is approximately 6

5   tumor cells / 100 ml of blood. Increase in tumor mass
might be expected to be proportional to an increase in
the frequency of the circulating tumor cells. If this
were found to be the case, methods available with this
level of sensitivity would facilitate both assessing

10   tumor load in patients with distant metastasis and also
assessing tumor load in patients with localized disease.
Detection of tumor cells in peripheral blood of patients
with localized disease has the potential not only to
detect a tumor at an earlier stage but also to provide

15   indications as to the potential invasiveness of the
tumor.

        Several studies report the presence of carcinoma
cells in leukopheresis products harvested from patients
with carcinoma of the breast for autologous peripheral

20   blood stem cell transplantation (Brugger W, et al.
(1994) Blood 83:636-640; Brockstein BE, et al. (1996) J
of Hematotherapy 5:617; Ross AA, et al. (1993) Blood
82:2605; Ross AA. (1998) J of Hematotherapy. 7:9-18;
Moss TJ, et al. (1994) J. Hematotherapy. 3:163-163).

25   These findings prompted criticism of the use of this
procedure for autologous transplantation since the tumor
cells in the transplant product have the potential to
establish metastasis (Racila E, et al. (1998) PNAS USA.
95:4589-4594). Additionally, it was found that

30   leukopheresis products were more likely to contain tumor
cells when obtained from individuals with disseminated
disease (Brugger et al., 1994, *supra*). These studies,

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however, do not report quantitative data, nor do they report that tumor cells can be found in peripheral blood of patients with localized disease. Given these observations, one may hypothesize that a highly

5 sensitive and quantitative test that counts the number of tumor cells in peripheral blood may be used to determine actual tumor load. To assess the feasibility of such testing, a sensitive cellular assay was developed which allows precise enumeration of

10 circulating carcinoma cells that is limited only by the blood volume to be tested.

It should be noted that a number of different cell analysis platforms can be used to identify and enumerate the enriched samples. Examples of such analytical

15 platforms are Immunicon's CellSpotter system, a magnetic cell immobilizer for manual observation of cells, and the CellTracks system, an automatic optical scanning magnetic cell immobilizer described in US patent applications 08/931,067 and 08/867,009 respectively.

20 Both of the aforementioned U.S. Patent Applications are incorporated by reference herein as disclosing the respective apparatus and methods for manual or automated quantitative and qualitative cell analysis.

Other analysis platforms include Laserscanning

25 Cytometry (Compucyte), bright field base image analysis (Chromavision), and Capillary volumetry (Biometric imaging).

The enumeration of circulating epithelial cells in blood using the methods and compositions of a preferred

30 embodiment of the present invention is achieved by immunomagnetic selection (enrichment) of epithelial cells from blood followed by the analysis of the samples

by multiparameter flowcytometry. The immunomagnetic
sample preparation is important for reducing sample
volume and obtaining a 10^4 fold enrichment of the target
(epithelial) cells. The reagents used for the
5 multiparameter flowcytometric analysis are optimized
such that epithelial cells are located in a unique
position in the multidimensional space created by the
listmode acquisition of two lightscatter and three
fluorescence parameters. These include 1) an antibody
10 against the pan-leucocyte antigen, CD45 to identify
leucocytes (non-tumor cells); a cell type specific or
nucleic acid dye which allows exclusion of residual red
blood cells, platelets and other non-nucleated events;
and 3) a biospecific reagent or antibody directed
15 against cytokeratin or an antibody having specificity
for an EpCAM epitope which differs from that used to
immunomagnetically select the cells.

It will be recognized by those skilled in the art
that the method of analysis of the enriched tumor cell
20 population will depend on the intended use of the
invention. For example, in screening for cancers or
monitoring for recurrence of disease, as described
hereinbelow, the numbers of circulating epithelial cells
can be very low. Since there is some "normal" level of
25 epithelial cells, (very likely introduced during
venipuncture), a method of analysis which identifies
epithelial cells as normal or tumor cells is desirable.
In that case, microscopy based analyses may prove to be
the most accurate. Such examination might also include
30 examination of morphology, identification of known tumor
markers and or oncogenes. Alternatively, in disease
states wherein the number of circulating epithelial

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cells far exceeds that observed in the normal
population, an analytical method which enumerates such
cells should be sufficient. The determination of
patient status according to the methods described herein

5   is made based on a statistical average of the number of
circulating rare cells present in the normal population.
Levels of circulating epithelial cells in the early
stage cancer patient and in patients with aggressive
metastatic cancer can also be statistically determined

10   as set forth herein.

     The following methods are provided to facilitate
the practice of the present invention.

15   **Patients.** With informed consent, 8-20 ml blood samples
were obtained from controls and patients with carcinoma
of the breast, prostate and colon. Blood was drawn from
some of these patients at several time points over a
period of one year. The blood samples were drawn into

20   Vacutainer tubes (Becton-Dickinson) containing EDTA as
anticoagulant. The samples were kept at room
temperature and processed within 24 hours after
collection. The circulating epithelial cells were
enumerated in peripheral blood samples from breast,

25   prostate and colon cancer patients and in normal
controls with no evidence of malignant disease. Date of
diagnosis, therapeutic interventions and clinical status
were retrieved from the patient's charts. The
institutional review board of the collaborating

30   institutions approved the protocol.

     Sample preparation. Monoclonal antibodies specific for

epithelial cell adhesion molecule (EpCAM) are broadly
reactive with tissue of epithelial cell origin (Stahel
RA, et al. Int J Cancer Suppl. 8:6-26 (1994); Momburg
F, et al. Cancer research. 47:2883-2891 (1987); Gaffey
MJ, et al. Am J Surg Path. 16:593-599 (1992)). The
GA73.3 or MJ37 EpCAM antibodies recognizing two
different epitopes on EpCAM (kindly provided by D Herlyn
(Herlyn D, et al. J Immunol Methods. 73:157-167
(1984)) Wistar Institute, Philadelphia, PA and MJ Mattes
(De Leij L, et al. Int J Cancer Suppl. 8:60-63 (1993))
Center for Molecular Medicine and Immunology, NJ) were
coupled to magnetic nanoparticles (ferrofluids) (Liberti
PA & Piccoli SP, United States Patent No. 5,512,332
(1996), Immunicon, Huntingdon Valley, PA). Blood was
incubated with the anti-EpCAM conjugated ferrofluid for
15 minutes in disposable tubes with an internal diameter
of 13 mm. The tubes were placed into a separator
composed of four opposing magnets for 10 minutes (QMS13,
Immunicon, Huntingdon Valley, PA). After separation,
the blood was aspirated and discarded. The tube was
taken out of the magnetic separator and the collected
fraction was resuspended from the walls of the vessel
with 2 ml of FACS permeabilization solution (BDIS, San
Jose, CA) and placed in the magnetic separator for 5
minutes. The solution was aspirated and discarded and
the cells were resuspended in 150 μl of cell buffer
(PBS, 1% BSA, 50mM EDTA, 0.1% sodium azide) to which
phycoerythrin (PE) conjugated anti-cytokeratin (CAM5.2
Monoclonal antibody) and Peridinin Chlorophyll Protein
(PerCP)-labeled CD45 were added at saturating
conditions. After incubation for 15 minutes, 2 ml of
cell buffer was added and the cell suspension was

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magnetically separated for 5 minutes. After discarding
the nonseparated suspension, the collected cells were
resuspended in 0.5 ml of the buffer to which the nucleic
acid dye used in the Procount system from BDIS, San

5   Jose, CA, was added according to manufacturer's
instructions. In some cases in which the EpCAM antibody
MJ37 was used on the ferrofluid, GA73.3 PE was used to
identify the selected epithelial cells. In these cases
no permeabilization of the cells is required. Reagents

10   for flowcytometry were kindly provided by BDIS, San
Jose, CA.

An exemplary method for determining the tissue
source of circulating epithelial cells employs
cytochemical and immunological identification

15   techniques. Primary monoclonal antibodies recognizing
cytokeratins 5, 6, 8, 18 (CK, 5D3, LP34, Novocastra),
MUC-1 glycoprotein (MUC-1, Ma695 Novocastra) or prostate
specific antigen (PSMA), clone J591 obtained from Dr.
Neil Bander (University of Texas Medical Center, Dallas,

20   Texas) was added to the slides after blocking non-
specific binding sites with 5% BSA for 30 minutes. The
samples were incubated for 20 minutes at room
temperature, washed twice in PBS for 5 minutes and then
exposed to secndary rabbit anti-mouse Ig (Z0259, Dako

25   Corp., Carpenteria, CA) for another 20 minutes. After
two more washes, the samples were incubated with
alkaline-phosphatase-anit-alkaline phosphatase (APAAP)
rabbit Ig complexes for 15 minutes. Finally, the
enzyme-substrate (New Fuchsin, Dako Corp. CA) was added

30   resulting in the development of red precipitates. The
nucleus was counterstained with hemotoxylin. The data
were recorded using a Kodak digital camera attached to a

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light microscope.   Data could be stored on CD for later

reference.

Sample analysis.   85% of the samples were analyzed on a

5   FACSCalibur flowcytometer (BDIS, San Jose, CA). The data

were acquired in listmode using a threshold on the

fluorescence of the nucleic acid dye.   Multiparameter

data analysis was performed using Paint-A-Gate^{Pro} (BDIS,

San Jose, CA).   Analysis criteria included size defined

10   by forward light scatter, granularity defined by

orthogonal light scatter, positive staining with the PE

labeled cytokeratin monoclonal antibody and no staining

with the PerCP labeled CD45 monoclonal antibody. For

each sample, the number of events present in the region

15   typical for epithelial cells was normalized to 10 ml of

blood.

The following examples are provided to facilitate

the practice of the present invention.   These examples

20   are not intended to limit the scope of the invention in

any way.

EXAMPLE 1

25   **Formulation of improved magnetic nanoparticles for the**

efficient isolation of rare cells from whole blood

Rare cells (e.g., tumor cells in patients with

epithelial derived tumors, fetal cells in maternal blood

or the like) can be present in frequencies below one

30   rare cell per ml of blood.   The number of blood smears

required to detect such rare cells is prohibitively

large.   Assuming 10 rare cells in 10 ml of blood, which

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corresponds to 10 tumor cells in 5-10 x 10^7 white blood cells (leukocytes), cells can be transferred to a microscope slide by cytocentrifugation or by settling, stained with an antibody specific for the rare cells of interest and read manually or automatically. The maximum number of cells that can be transferred to one slide is about 500,000 cells which means 100-200 slides are required to process 10 ml of blood. The time required for analysis by this approach makes it impractical and economically unfeasible. Consequently, enrichment methods such as sample volume reduction and removal of erythrocytes and platelets by density gradient separation or erythrocyte lysis procedures are used for isolating rare cells so as to significantly reduce the number of slides to be analyzed.

As noted above, magnetic enrichment is the preferred method for cell separations and, ideally, the nanoparticles employed for this purpose should not have to be removed prior to analysis. Accordingly, the nanoparticles should be small enough so as not to interfere with analytical measurements, i.e. below about 250 nm. Most preferably, the nanoparticles are below 220 nm so as to make them filter sterilizable. Furthermore, the nanoparticle should be large enough and magnetically responsive enough to permit cell separation from simple laboratory tubes, i.e., test tubes, centrifuge tubes, vacutainers and the like in external gradient magnetic separators. Again, as previously noted internal gradient devices are cumbersome, costly and inefficient for the recovery of rare cells. Also, the nanoparticles and magnetic device should give high and reproducible recovery with low non-specific binding. US Patent No.

40

5,597,531 describes the synthesis of highly magnetic
particles, referred to as direct coated (DC) particles
which have many of these characteristics. These
nanoparticles are composed of quasispherical

5   agglomerates of crystalline magnetite or other magnetic
oxides which are coated with polymers or proteins (based
coated magnetic particles). Because of their structure
(magnetic core and polymer coat where the core diameter
is >>> than the thickness of the coat) they are about

10   80-85% magnetic mass. The non-specific bindings of
these nanoparticles are in the range of 5-8 % and they
are, therefore, not very practical for rare cell
separations. Thus if one is enriching cells present at
one cell per ml then at 80% capture efficiency, the best

15   result to be expected using 10 mls of whole blood
(considering leukocytes alone) would be 8 cells
recovered in a total of 4 million, i.e. a 16-17 fold
enrichment. The magnetic particles described in U.S.
Patent 5,597,531 do, however, have the appropriate

20   magnetic properties to perform separations with open
field separators and from simple laboratory tubes.
Further, their mean size is well under the limit
suggested above and, hence, they do not interfere with
various analytical procedures. Based on extensive

25   studies with those materials, the major contributing
factor to non-specific binding to cells was discovered
to be the presence of bare crystalline iron oxides on
the nanoparticles due to incomplete coating. Such
incompletely coated crystals have a sufficiently high

30   positive charge at physiological pH that they are very
likely to bind strongly to biological macromolecules,
such as negatively charged sialic acid on cell surfaces.

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An improved method for making particles is described in
U.S. Patent No. 5,698,271. These materials are an
improvement over those disclosed in the '531 patent in
that the process includes a high temperature coating
5   step which markedly increases the level of coating.
Nanoparticles made with bovine serum albumin (BSA)
coating using this process, for example, have a 3-5-fold
lower non-specific binding characteristic for cells when
compared to the DC-BSA materials of US Patent 5,579,531.
10   This decrease in non-specific binding has been shown to
be directly due to the increased level of BSA coating
material. When such nanoparticles were treated so as to
remove BSA coating, non-specific binding returns to high
levels. It was thus determined that a direct
15   relationship exists between the amount of BSA coated on
iron oxide crystal surfaces and the nonspecific binding
of cells. Typically, the non-specific binding of cells
from whole blood with these particles was 0.3% which is
significantly better than those produced from US Patent
20   5,579,531. Thus, from 10 mls of whole blood there would
be about 200,000 non-target cells that would also be
isolated with the cells targeted for enrichment.

        In addition to the non-specific binding problem, to
be addressed further below, it was found that when
25   different lots of magnetic particles, manufactured as
described in US Patents Nos. 5,579,531 and 5,698,271
were used in rare cell depletions or enrichments,
recoveries were inconsistent. Sometimes recoveries were
85-95% and other times they could be 40-50% using the
30   same model system. As the process for manufacturing
these materials results in a size dispersion of
considerable range (30nm to 220 nm), it was suspected

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and confirmed that the size distribution and
particularly the presence of small nanoparticles
markedly affected target recovery. Since small
nanoparticles (30 to 70nm) will diffuse more readily
5   they will preferentially label cells compared with their
.larger counterparts. When very high gradients are used,
such as in internal gradient columns, the performance of
these materials regardless of size make little
difference. On the other hand, when using external
10   gradients, or gradients of lesser magnitude than can be
generated on microbead or steel wool columns, the
occupancy of small nanoparticles on cells has a
significant effect. This was conclusively shown to be
the case by fractionating DC nanoparticles and studying
15   the effects on recovery. Based on these studies and
other optimization experiments, means for fractionating
nanoparticles magnetically or on columns was established
where base coated magnetic particles could be prepared
that were devoid of excessively small or large
20   nanoparticles. For example, base coated particles of
mean diameter 100nm can be produced which contain at
best trace amounts of material under 80 nm or over 130
nm. Similarly material of about 120 nm can be made with
no appreciable material under 90-95 nm and over 160 nm.
25   Such materials performed optimally with regard to
recovery and could be made sub-optimal by the inclusion
of 60-70 nm nanoparticles. The preferred particle size
range for use in practicing this invention is 90-150 nm
for base coated magnetic particles, e.g., BSA-coated
30   magnetite. Particles falling within this preferred
range may be obtained using the procedure described by
Liberti et al. In Fine Particles Science and Technology,

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777-90, E. Pelizzetti (ed.) (1996).

To further address the non-specific binding
problem, several routes for making antibody conjugated
direct nanoparticles were attempted. Monoclonal
5   antibody specific for rare cells can be directly coupled
to, for example, the BSA base coating on the DC magnetic
particles by standard heterobifunctional chemistry
(referred to herein as direct coupling method).
Heterobiofunctional linkers used for these purposes
10   include sulfo-MCCC and sulfosuccinimidil-4-
[maleimidomethyl]cyclohexane-1-carboxylate. In another
approach, biotinylated monoclonal antibodies can be
coupled to streptavidin which has been coupled to the
base coated particles. This conjugate method is
15   referred to herein as a piggyback method. In this
process, streptavidin is coupled to the base coated
magnetic particles by the same chemistry as the direct
coupling method. In one piggyback coupling method,
monobiotinylated antibody is allowed to react with
20   streptavidin magnetic particles for 1 hour and then the
remaining streptavidin binding sites quenched with free
biotin. It is important to quench the remaining
streptavidin sites after antibody coupling to prevent
binding of any biotinylated antibody to magnetic
25   particles during isolation of rare cells or the cell
analysis step. Furthermore, it has been shown that this
means for quenching streptavidin is effective for
counteracting non-specific binding. Incubation of such
materials under a variety of conditions with
30   biotinylated fluorescent macromolecules results in no
bound fluorescence. For comparison, anti-EpCAM antibody
(GA73.3 obtained from the Wistar Institute,

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Philadelphia, Pa.) was coupled to magnetic particles by
both methods. Both magnetic particles were then
compared for the selection of cells from the colon tumor
cell line (Colo-205) spiked into whole blood as well as
5   for the non-specific binding (NSB) or carry-over of
leukocytes. The leukocytes present in the final sample
were a combination of leukocytes non-specifically bound
to magnetic particles and carry-over of cells from the
wash steps. Note that following magnetic separation, it
10   is necessary to wash away any cells which were in
contact with the tube at the start of the separation or
that were transported non-magnetically during the
separation process. The following table shows the
comparison of those two magnetic particles.
15

Magnetic particles	Recovery of spiked Colo-205 cells (%)	NSB and carry over leukocytes (%)
EpCAM antibody directly coupled to magnetic particles (lot.# 120325-1)	78-82	0.1 - 0.3
EpCAM antibody coupled to magnetic particles by piggyback method (lot.# 120607-2)	67 - 78	0.05 - 0.1

The first thing noted is that merely coupling
antibody or Streptavidin to BSA base particles

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significantly reduces non-specific binding (data not
shown). This is believed to be due to decreasing the
accessability of "bare" crystal surfaces to cells for
binding. The above table demonstrates that the recovery
of spiked cells is comparable for both types of magnetic
particles. However, the non-specific binding of
leukocytes was 3-fold higher when using the direct
antibody coupled magnetic particles. This difference,
albeit relatively small, becomes significant when a
large volume of blood is processed and analyzed. A
reasonable explanation based on many supporting
observations for the difference between the two types of
magnetic particles is that there are more layers of
protein on magnetic particles synthesized using the
piggyback coupling method. The surface of the magnetic
crystals are thus coated more extensively with multiple
layers of protein and appear to be sterically
"protected". This prevents binding of non-target cells
to the magnetic particles.

        In the piggyback coupling method, a limited number
of streptavidin binding sites on the magnetic particles
are occupied with biotin-antibody and the remainder are
saturated with free biotin by the quench process
described above. In yet another coupling method, the
excess streptavidin binding sites were quenched and
saturated with monobiotin-BSA instead of free biotin.
The rationale for this approach is that quenching with
monobiotin BSA should further sterically inhibit cells
from coming in contact with uncoated regions of the
nanoparticles, i.e. give better coverage of the
nanoparticles. It was shown by carbon analysis that
this process increases the amount of protein coupled to

the particles.   The two magnetic particle preparations
were compared in experiments assessing recovery of
spiked Colo 205 from whole blood and for non-specific
binding of leukocytes. The results are presented in the
5   following table.

Magnetic particles	Recovery of Colo 205 cells (%)	NSB and carry over leukocytes (%)
EpCAM antibody coupled magnetic particles – quenched excess streptavidin sites with free biotin (lot. # 131022-1)	93 87 85	0.08 0.1 0.1
EpCAM antibody coupled magnetic particles – quenched excess streptavidin sites with biotin-BSA (lot. # 131022-2)	87 83 85	0.01 0.03 0.02

Monobiotin-BSA may be prepared by conjugating a
limited amount of biotin to BSA, such that 30- 40% of
the resultant product has no bound biotin.

In summary, magnetic particles having a homogeneous
size distribution and biotin-BSA quenched streptavidin
30   binding sites performed extremely well in the assay
methods of the present invention. A good recovery of
the spiked epithelial tumor cells and almost an order of
magnitude reduction in nonspecific binding is obtained
using these particles, compared with the biotin blocked
35   nanoparticles. Thus, these materials and the results
obtained with them define a very useful product which

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can be further optimized. The improved ferrofluid
product is made as magnetic as possible, is coated so as
to exclude all possible interactions of the magnetic
core with any substances in blood including cells

5   (presumably coated with a nonporous monolayer) and are
well defined in its size range and distribution. In the
preferred situation, a coat material is used which does
not interact with biological materials. Where such
interactions are unavoidable, a means for blocking them

10   is required. For a material to be as magnetic as
possible, those produced as described in US Patent Nos.
5,579,531 and 5,698,271 are preferred starting
materials. They are preferable because they are
composed of large magnetic cores with an apparent but

15   not complete monolayer of base coating material. For a
100 nm nanoparticle coated with BSA, the core will be
about 90 nm of an appropriate magnetic oxide such as
magnetite. Such nanoparticles because of the relative
size of the cores and coat material are clearly as

20   magnetic as is possible. This is apparent if one
considers that the function of the coating is to keep
the nanoparticles from undesired interactions with each
other which would lead to macroscopic agglomeration.
The coating also promotes sufficient interactions with

25   solvent molecules so as to maintain colloidal behavior
and provides a convenient chemical means for coupling.
The nanoparticles of US Patent Nos. 5,579,531 and
5,698,271 are also preferred as a starting material as
they have sufficient monolayer coating wherein "holes"

30   in the monolayer can be filled in several ways, viz.,
sterically and physically. Clearly any coating that
promotes the effective complete coverage of the magnetic

core, so as to inhibit interactions of the core material with blood components or any other non-specific effects in any other system would be suitable. The less mass such a coating might add to the nanoparticles the

5   better, so as to maximize the magnetic mass to nanoparticle mass ratio.

EXAMPLE 2

Enumeration of circulating epithelial cells in patients

10   **treated for metastatic Breast Cancer**

Figure 1 shows the results obtained when tumor cells spiked into whole blood are isolated using the assay methods of the present invention. Panel A shows analysis by microscopy and panel B shows analysis

15   results obtained using flow cytometry. Fig. 2 shows three examples of the flowcytometric analysis of 10 ml blood samples obtained from one patient with metastatic breast carcinoma at three time points, and includes the correlative display of the anti-leukocyte versus anti-

20   epithelial cell antibodies of the flowcytometric analysis. In Fig. 2, Panel A, 14 events are detected and are present in the location typical for epithelial cells. In Panel B, 108 epithelial cells are detected and in Panel C 1036 epithelial cells are detected.

25   The number of events passing the threshold set on the nucleic acid dye in the analysis of the 10-ml blood sample varied between 5,000 and 50,000 events. These events consist of cellular debris and leukocytes. In analyzing the blood of 32 controls, the number of

30   events present in the region typical for epithelial cells ranged from 0 - 4 / 10 ml of blood (mean = 1.0, SD = 1.2).

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Eight breast cancer patients had active metastatic
disease during the period of study. In these patients,
the number of epithelial cells in 10 ml of blood varied
within the range of 0 to 1036. The activity of the

5   disease was assessed by subjective criteria, i.e. bone
pain, dyspnea etc. and objective criteria, X-rays, bone
scans, CT scan, MRI and lymph node size. Patients were
classified in categories 0 through 4,as set out in Table
1.

10

TABLE I
Classification of patients according to clinical activity of the
disease after surgical intervention

	Category	Criteria
15	0	No evidence of disease at any time point after surgical intervention
	1	Evidence of disease at one time point after surgical intervention
	2	Evidence of disease under control
20	3	Active progressive disease
	4	Life threatening disease

The dynamics of epithelial cell counts in the
blood of 8 patients with metastatic disease are

25   presented in Fig. 3. The shaded area in the plots
indicates the range at which positive events were
detected in the controls. The plots also indicate when
chemotherapy was administered. Figure 3, panel A shows
a patient with life threatening disease and 200

30   epithelial cells / 10 ml of blood at the time' she
entered the study. High dose adriamycine reduced the
number within the normal range, but it rose again after
adriamycine was discontinued. After a second course of
adriamycine, the number of epithelial cells dropped

35   significantly, but was still above the normal range.
Fig. 3, Panel B shows the course of one patient over a
period of 43 weeks. The patient was asymptomatic at the

start of the study but was known to have bone metastasis
in the past. Epithelial cells were detected above
normal levels and steadily increased during the period
studied. A brief decline in the number of epithelial

5   cells was found after a course of high dose adriamycine
was administered. The activity of disease in this
patient clearly increased during this period. In Fig.
3, Panels C and D, two patients are shown with less
disease activity. In these patients, the changes in the

10   number of epithelial cells over time also reflected the
changes in the activity of the disease. In the patients
shown in Panels E and F, the number of peripheral blood
epithelial cells increased at the last time point
studied while the patients still were without symptoms.

15   In the case shown in Panel G, no epithelial
cells were detected at the first time point studied
which was three years after breast cancer surgery
(T2N1M0). Four weeks later, 50 epithelial cells in 10 ml
of blood were detected by flowcytometry. The patient at

20   this time had no clinical signs of disease recurrence.
An additional blood sample was analyzed to obtain
morphological confirmation that the cells detected by
flowcytometry had features consistent with those of
malignant cells.

25   Figure 4A shows two cells with a large nuclear
to cytoplasmic ratio and which positively stain with
Cytokeratin, both features being consistent with tumor
cells of epithelial cell origin. Four weeks after this
finding, the patient had an axillary lymph node biopsy.

30   Cells obtained from the biopsy proved to be of malignant
origin. Although an X-ray at this time did not show
signs of pulmonary metastasis, a CT scan performed two

weeks later showed evidence of pulmonary metastasis.
The patient had no symptoms from the pulmonary
metastasis. The patient reacted well to Vinorelbine as
measured by the disappearance of the axillary lymphnode

5   involvement. The peripheral blood epithelial cell
number dropped to levels just above the normal range.
Twenty-eight weeks after initiation of the treatment,
the peripheral blood epithelial cell number increased
and by physical examination, the axillary node increased

10   in size. The number of peripheral blood epithelial
cells in these 8 patients with metastatic disease of
carcinoma of the breast clearly reflected the activity
of the disease and the response to treatment or the lack
thereof during the time period studied.

15   The experiments described above were performed
using colloidal magnetic nanoparticles. In this
example, the efficiency of larger size magnetic beads
for the selection of tumor cells present at a low
frequency in blood was also evaluated to determine

20   whether micron size beads can also be used to select
tumor cells even though as described above, nanometer
size magnetic particles are considered preferable for
this application.

          As mentioned previously, disadvantages are

25   encountered with the use of larger size beads. These
are: (i) the beads are too large to diffuse thus
collisions of the beads with target cells present at a
low frequency requires mixing (ii) the beads settle
very fast, furthering the need for continuous mixing and

30   (iii) large size beads cluster around cells and obscure
analysis. Accordingly the large size beads need to be
removed from the cell surface prior to visualization or

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analysis. In accordance with the present invention, it has been found that the efficiency of cell selection with larger beads can be improved by increasing the concentration of beads and increasing the incubation

5   time with continuous mixing to facilitate binding to rare target cells. In this example, 2.8μm Dynal anti-epithelial cell beads (Dynal, NY) were used to test the efficiency of tumor cell selection from blood in a model study under optimum conditions for large beads.

10   These beads are conjugated with a monoclonal antibody specific for epithelial tumor cells. A known number of tumor cells (cancer cell line) were spiked into normal blood to determine the recovery after selection with beads. The tumor cells were prelabeled with a

15   fluorescent dye to differentiate them from blood cells during detection. The protocol was followed as recommended by the manufacturer.

Whole blood (5 ml) was added to a 15 ml polystyrene centrifuge tube followed by the addition of

20   20 ± 3 fluorescently labeled SKBR-3 (breast cancer cell line) cells. SKBR-3 cells were prestained with a nucleic acid staining dye (Hoescht) to allow detection after the selection by beads. The blood was diluted with 5ml of Dulbecco's PBS containing 5 mM EDTA and mixed with the

25   diluted blood for 15 minutes at 4°C on a rocker. 100μl of Dynal anti-epithelial cell beads containing 50 x 10⁶ beads were added to the blood sample and incubated for 30 minutes at 4°C with mixing on a rocker. Note that the number of beads used were similar to total white blood

30   cells i.e. one bead per white cell. The magnetically labeled cells were separated by placing the sample tube into Dynal MPC magnetic separator for 6 minutes.

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After aspirating the supernatant, the collected cells were resuspended in 3ml of Dulbecco's PBS containing 0.1% BSA. The sample tube was placed back into Dynal's MPC for 6 minutes to remove any carry-over

5   blood cells. The magnetically bound cells were resuspended in 200 μl of Dulbecco's PBS containing 0.1% BSA after aspiration of the supernatant.

The final sample, containing selected tumor cells, non-specifically bound blood cells and excess

10   free magnetic beads, was spotted onto an immunofluorescent slide to detect tumor cells. The 200μl sample was spotted into 10 different wells to disperse free magnetic beads. The fluorescently stained tumor cells present in each well were counted using a

15   fluorescent microscope. The results are shown in the Table II:

TABLE II

Experiment No.	Tumor cells recovered	% Recovery
1	16	80
2	17	85
3	10	50
4	11	54

The results show that, on average, 67% of the

25   spiked tumor cells were recovered from blood by Dynal magnetic beads. This suggests that tumor cells present in blood can be selected efficiently with larger size magnetic beads under optimum conditions. In this example, however, only the selection of tumor cells from

30   blood was evaluated without performing any analysis. Further the efficiency of recovery could be determined because cells were prelabeled with a strong fluorescent

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dye. The final sample (200µl) contained 50 x10^6 beads in addition to selected tumor cells (10-17) and non-specifically bound leukocytes. The size of the beads (2.8um) is similar to that of certain blood cells and

5   occupied most of the surface area on the slide. Therefore, to obtain recovery data, the sample had to be spotted onto several wells in order to sufficiently disperse free magnetic beads so as to allow for detection of recovered tumor cells.

10   There were also many beads on cell surfaces which precludes viewing and staining of selected tumor cells for further analysis. In this example, tumor cells were prestained with a fluorescent nucleic acid dye and further staining was not necessary for detection.

15   However it is often desirable to identify the tissue of origin of the magnetic bead-bound cells. Such identification is performed using labeled antibodies to detect and characterize tumor cells present in clinical samples. Accordingly, beads have to be removed from

20   cell surfaces and separated from the sample following target cell selection, i.e. before analysis. This is not the case with nanosize magnetic particles because their size does not interfere with cell analysis.

              In summary, this example shows that large

25   magnetic beads may also be utilized in the methods disclosed herein for the efficient isolation of circulating tumor cells.

              There are several methods available to release beads from cell surfaces which do not significantly

30   damage isolated cells. One method is to displace antibody from the cell surface by adding an excess specific competing reagent in excess which has higher

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affinity for the involved antigen or antibody. This
type of mechanism is used to release beads from CD34
selected cells in clinical applications using a peptide
(Baxter Isolex 300). The peptide competes with CD34
5   antigen for binding to antibody on beads and releases
the antibody-bead complex from cells. Another method
employs a reversible chemical linker between beads and
antibodies.

The chemical linker can be inserted during
10   the conjugation of antibodies to magnetic beads. The
chemical link can be cleaved under appropriate
conditions to release beads from antibodies. One of
the methods currently in use employs a nucleic acid
linker to link antibodies to magnetic beads. The
15   nucleic acid linker is a polynucleotide and can be
hydrolyzed specifically using DNAse enzyme. Following
hydrolysis of the nucleotide bonds present in the
nucleic acid linker, the beads are released from the
antibodies which remain bound to cells. The released
20   beads can be removed from cell suspension by magnetic
separation. The cells which are freed from beads can
be used for further analysis by microscopy or flow
cytometry.

This example demonstrates that larger size
25   magnetic beads can also be used to isolate tumor cells
from blood, provided they are used in high enough
concentration to label cells and are then released
from cells before analysis.

30

EXAMPLE 3

Enumeration of circulating epithelial cells in patients
with no evidence of disease after surgery for carcinoma
of the breast with curative intent

5   Peripheral blood of 37 patients between 1 and 20
years after surgery was examined for the presence of
epithelial cells by flowcytometry. Up to 7 peripheral
blood samples were taken over a one-year period from
these patients. In Table III, each of the patients is
10   listed and sorted according to the TNM (tumor, node, and
metastasis) stage at the time of surgery followed by the
years after surgery. Table III also shows whether or
not the patient received treatment (either chemotherapy
or hormonal therapy) during the period studied. In 3 of
15   6 patients with evidence of distant metastasis in the
past, but in complete remission at the time of study,
epithelial cells were found in the blood at a higher
frequency than that found in the control group.
Circulating epithelial cells were also found in 9 of 31
20   patients with no evidence of distant metastasis.

        The low number of events present in the region
typical for epithelial cells by flowcytometry in these 9
patients does not warrant identifying these events as
tumor cells. Cytology obtained by placing the
25   immunomagnetically selected cells on a slide greatly
aids in the assessment of their identity as is
illustrated in Fig. 4. Fig. 4, panel A, shows two cells
staining positive for cytokeratin and obtained from a
patient with no evidence of metastatic disease at the
30   time the blood was drawn. Panel B shows a cell from a
patient with metastatic disease in the past but in
complete remission. In Panels C and D, two cells are

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shown isolated from the blood of patient 25 at time
point 6. The cell shown in Panel C has features
consistent with malignancy whereas the cell in Panel D
has the appearance of a normal squamous epithelial cell.

TABLE III

Number of epithelial cells identified by flowcytometry in 10 ml of peripheral blood of patients with no evidence of disease after surgery for carcinoma of the breast with curative intent and 32 controls.

Patient Number	TNM	Ys	Tx	1	2	3	4	5	6	7
1	$T_3N_1M_1$	9	–	2	29					
2	$T_3N_1M_1$	16	H	0						
3	$T_2N_1M_1$	7	CT	10	7	5	6	4	8	7
4	$T_2N_1M_1$	10	CT	1	0	0	1	2		
5	$T_2N_1M_1$	10	H	6						
6	$T_2N_1M_1$	20	H	12	2					
7	$T_3N_1M_0$	1	H	0						
8	$T_3N_1M_0$	2	CT	0	0	0				
9	$T_3N_1M_0$	2	CT	0	0	1	0			
10	$T_3N_1M_0$	3	H	3						
11	$T_3N_1M_0$	3	H	5	4	0	6			
12	$T_3N_1M_0$	3	H	3	0					
13	$T_3N_1M_0$	6	CT	6	0					
14	$T_3N_1M_0$	6	H	1	1					
15	$T_3N_1M_0$	7	H	1	3	3				
16	$T_3N_1M_0$	3	H	0						
17	$T_2N_1M_0$	17	H	4						
18	$T_3N_0M_0$	3	–	5						
19	$T_3N_0M_0$	5	H	1						
20	$T_3N_0M_0$	8	H	0	6	8				
21	$T_2M_0M_0$	<1	–	0						
22	$T_2N_0M_0$	<1	H	0						
23	$T_2N_0M_0$	1	H	0						
24	$T_2N_0M_0$	1	–	4						
25	$T_2N_0M_0$	2	CT	3	5	1	3	6	2	
26	$T_2N_0M_0$	3	CT	2	6	3	1	1	0	5
27	$T_2N_0M_0$	6	H	18						
28	$T_2N_0M_0$	6	H	2	1					
29	$T_2N_0M_0$	7	H	8	4	2				
30	$T_2N_0M_0$	8	H	0	1					
31	$T_2N_0M_0$	8	H	0	6	8				
32	$T_2N_0M_0$	11	H	2						
33	$T_2N_0M_0$	20	H	4						
34	$T_1N_0M_0$	<1	H	0						
35	$T_1N_0M_0$	2	H	0						
36	$T_1N_0M_0$	17	–	0						
37	$T_?N_0M_0$	13	H	0						

N3 controls

2   min 0

max 4
mean 1.0   M ± 2SD 3.5

TNM = Tumor, Node, Metastasis
Ys = years after primary surgery
Tx = therapy, CT = chemotherapy, H = hormonal therapy, -
= no therapy
1,2,3,4,5,6,7 = subsequent time point at which the
number of epithelial cells was determined in years

EXAMPLE 4

**Enumeration of circulating epithelial cells in patients
diagnosed with breast cancer before surgical
intervention.**

Table IV summarizes the results obtained
following similar clinical trials in which 13 controls
and 30 patients with breast cancer were assessed using
the assay of the invention. In control individuals the
number of epithelial cells in 20 ml of blood ranged from
0-5 (mean 1.5 ± 1.8). In contrast, there was an average
of 15.9 ± 17.4 epithelial cells in the 20 ml blood
samples of 14 patients with organ-confined carcinoma of
the breast (patients classified as $T_xN_oM_o$), 47.4 ± 52.3
in those with nodal involvement, and 122 ±140 in those
with distant metastases. The difference between the
control group and patients with carcinoma of the breast,
with or without metastasis, was highly significant
[P,0.001 by multiparameter analysis (Kruskal-Wallis)].
The difference between the organ-confined and the
distant metastatic group was 0.009(t test). The number
of epithelial cells in patients with organ-confined
breast cancer was above the cut-off point (mean value
plus 3 SD in the control group = 6.9) in 12 of 14 cases.
Moreover, no individual in the control group had more
than 5 events classified as epithelial cells, and only 2

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of the 14 patients with organ-confined breast cancer had
<7 such events.

Table IV

Summary of clinical data

Number	Healthy Control	No detectable spread	Spread to lymphnodes only	Distant metastasis
1	0	0	7	20
2	0	4	8	20
3	0	7	14	20
4	0	8	93	23
5	0	8	115	54
6	0	8		62
7	0	12		99
8	2	13		135
9	2	14		152
10	4	16		304
11	4	18		456
12	5	19		
13		24		
14		72		
n	12	14	5	11
mean	1.5	15.9	47.7	122.5

Flowcytometry was used to analyze the positive events
obtained from 20 ml of blood from control individuals
and from women with breast carcinoma. The numbers of
epithelial cells in the blood of controls are
statistically different by t test ($P \leq 0.01$) and by
Kruskall-Wallis nonparametric analysis ($P<0.001$) from
each of the three groups of the breast cancer patients.
The data in this table were used to establish a
preliminary cut-off value for positive samples. This

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value was determined by averaging the number of circulating epithelial cells in the normal controls (n =13) and then adding three times the SD. The average was 1.5 and the SD is 1.8. Cut-off: 1.5 + 5.4 = 6.9. There is no statistical difference between male and female controls.

EXAMPLE 5

DISEASE ACTIVITY IS CORRELATABLE WITH NUMBER OF CIRCULATING EPITHELIAL CELLS IN PROSTATE CANCER PATIENTS

Three patients with metastatic disease of the prostate were assessed for the presence of circulating epithelial cells in their blood following chemotherapeutic treatment. The results are presented in Fig. 5. The data reveal that an increase in circulating epithelial cells in the blood is correlatable with disease activity. Also in three patients with no detectable spread of the cancer, epithelial cells were found in 20 ml of the peripheral blood (16 cells ± 4). As shown in Table V, the number of epithelial cells in the blood of prostate cancer patients was statistically different (P less than 0.001) than normal controls.

TABLE V

Number of Epithelial Cells Per 20 ml Blood	PSA Level (μg/ml)	Gleason Grade
4	6.0	3 + 4 =7
12	6.5	3 + 4 =7
12	11.2	3 + 3 =6
16	26.0	4 + 4 =8
20		
24	5.6	3 + 4 =7
28		

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Control blood samples were obtained from normal
individuals, individuals known to have benign tumors and
those patients with inflammatory diseases. Based on
5   statistical data, the results reveal that a cut-off
point of approximately 6.8 cells per 20 ml of blood was
useful as a diagnostic marker for prostate cancer.

10   EXAMPLE 6
DISEASE ACTIVITY IS CORRELATABLE WITH NUMBER OF
CIRCULATING EPITHELIAL CELLS IN COLON CANCER PATIENTS
        The assay method of the present invention may be
used to advantage in the assessment of patients with a
15   variety of different cancer types. To illustrate, the
method was also used to assess circulating epithelial
levels in patients with colon cancer. Colon cancer
patients without evidence of metastases were evaluated
for the presence of circulating epithelial cells before
20   and after surgery. The results are shown in Fig. 6 and
summarized in Table VI. The data reveal that the number
of circulating epithelial cells in colon cancer patients
is greater prior to surgical intervention.

TABLE VI
25   **CIRCULATING EPITHELIAL CELLS IN COLON CANCER PATIENTS WITHOUT**
EVIDENCE OF METASTASES

TIME OF TESTING	NUMBER OF PATIENTS TESTED	CIRCULATING EPITHELIAL CELLS DETECTED BY FLOW CYTOMETRY IN 10 ml OF BLOOD	
		MEAN ± SEM	RANGE
Before surgery	12	42.3 ± 22.0	0 - 234
After surgery	25	2.7 ± 0.7	0 - 15

35   Table VII and Fig. 7 depict data obtained when
colon cancer patients with evidence of metastases were

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assessed for the presence and number of circulating

epithelial cells. The results revealed that the number

of epithelial cells in peripheral blood is larger in

patients with metastatic disease as compared to local

5   disease after surgery. The results further show that

the extent of metastatic disease may be correlated with

the number of circulating epithelial cells.

TABLE VII

10   CIRCULATING EPITHELIAL CELLS IN COLON CANCER PATIENTS WITH EVIDENCE
OF METASTASES

METASTATIC STATUS OF PATIENTS TESTED	NUMBER OF PATIENTS TESTED	CIRCULATING EPITHELIAL CELLS DETECTED BY FLOW CYTOMETRY IN 10 ML OF BLOOD	
		MEAN ± SD	RANGE
REGIONAL	11	3.7 ± 0.6	1 - 6
DISTANT, SOLITARY	16	7.6 ± 2.0	0 - 21
DISTANT, MULTIPLE	8	54.0 ± 25.1	5 - 200
NORMAL CONTROL	32	1.0 ± 0.2	0 - 4

The examples above demonstrate the highly-

25   significant differences in the number of circulating

epithelial cells between healthy individuals and

patients with breast, prostate and colon cancer. In

addition, significant differences in the number of

circulating epithelial cells were found between patients

30   with no detectable spread, spread to local lymph nodes

and distant metastasis (Racila et al., (1998), *supra*).

Additionally, the number of epithelial cells in the

blood of patients after surgical removal of a primary

carcinoma of the breast was monitored over a one-year

35   period. In some of these patients residual disease was

detected. See Figure 7. In patients with metastatic

disease, the changes in peripheral blood tumor cell

count correlated with the tumor load and response to

treatment. The results of these studies reveal the
potential of the cell-based assay of the present
invention as an objective non-invasive tool to detect
the presence of malignant disease and measure the
5   activity of the disease. Cellular morphology and
immunophenotype reveal the malignant nature of the
isolated cells.

EXAMPLE 7

10   ## TISSUE SOURCE IDENTIFICATION OF ISOLATED EPITHELIAL
CELLS

All of the forementioned studies in patients reveal
that there is an excess of epithelial cells in patients
who have cancer, compared to normal individuals or
15   patients without cancerous diseases, including benign
tumors. It is essential, however, to prove that these
excess epithelial cells are, in fact, cancer cells.
This was accomplished by performing an experiment in
which immunomagneticallly purified epithelial cells from
20   patients with or without cancer were cytospun onto a
glass slide and treated with anti-mucin. In addition,
normal epithelial cells which were obtained from
foreskin and blood from normal individuals, both used as
controls, were also cytospun. It is significant that
25   the slides were coded and examined "blinded", that the
observer had training in pathology and that normal
epithelial cells were included. As can be seen in
Figure 4, there is a marked difference between the
cancer cells versus normal epithelial cells. Normal
30   epithelial cells have a low nuclear to cytoplasmic
ratio, i.e., there is abundant cytoplasm and a
relatively small nucleus. The nucleus shows a smooth

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distribution of chromatin. The cells do not stain with
anti-mucin. In contrast, cells from two patients with
breast cancer have very large nuclei and a small rim of
cytoplasm. Additionally, the chromatin is disorganized
as shown by the dark patches in the nucleus and the
cells stain intensively with anti-mucin. The same is
observed in cells from two patients with prostate
cancer. A physician trained in pathology was shown
coded slides from patients with and without cancer
(total of 21 slides). The pathology-trained physician
correctly identified bloods from all the controls as not
having cancer cells and displayed no-intraobserved error
when shown slides twice. In the cases of two patients
with prostate cancer, tumor cells were not seen in the
study. One slide was re-examined and tumor cells were
observed. The cause of this discrepancy appears to be
the amount of time spent scanning the cell smear. In
summary, the cytomorphology and immunophenotype indicate
that the excess epithelial cells present in the blood in
patients with cancer are indeed cancer cells.

The experiments described above indicated that
the methods disclosed herein enable the detection of
cancer cells in the blood of patients with early tumors.
Indeed, in 25 of 27 patients who were clinically
determined to have organ-confined disease (early stage
cancer), we detected the presence of cancer cells in the
blood. This means that the assay should detect cancer
cells much earlier in those solid tumors that are
normally detected late (10^9-10^{10} tumor cells). Morever,
the test should allow detection of breast and prostate
cancer earlier, perhaps before detection of a primary
tumor by conventional means. The organ-origin of tumor

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cells in the blood for prostate can be established by
staining with anti-prostate specific membrane antigen
(PMSA), anti-PSA (prostate specific antigen), or other
antibodies specific to the prostate in male subjects.

5   For breast carcinoma in female patients, staining with
anti-mammoglobin, anti-progesterone receptor, anti-
estrogen receptor and anti-milk fat globulin antigen I
and II will indicate a breast origin of tumor.

Our test should detect carcinoma cells from

10   other organs, e.g., pancreas, esophagus, colon, stomach,
lung, ovary, kidney, etc. The following table shows
examples in which excess epithelial cells were observed
in several patients with carcinomas other that the
breast and prostate.

TABLE VIII

NUMBER OF CELLS PER 20 ML BLOOD	CANCER DIAGNOSIS
8	Uterus adenocarcinoma (Stage 1B)
11	Head and Neck adenocarcinoma
15	Lung small undifferentiated
14	Neck Squamous cell carcinoma

15

20

25

Each of the carcinomas described in the table
above express tissue specific antigens whose

30   corresponding antibodies can be used to determine the
organ-origin of the circulating tumor cells.

The blood test of the invention can also be
used to detect cancer cells in patients previously
treated successfully for cancer and now in long term

35   complete remission. Indeed circulating epithelial
cells, i.e., dormant tumor cells, have been detected in
patients treated five or more years previously and who
appear to be clincally free of tumor. This explains why

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recurrence in patients can occur many years, even
decades after apparently successful treatment. In fact,
accumulating evidence suggests that the recurrence rate
of breast cancer is at slow steady rate 10-12 years

5   after mastectomy.

EXAMPLE 8

DETECTION OF TUMOR CELLS IN THE BLODD OF A PATIENT WITH HIGH PSA LEVELS AND A NEGATIVE BIOPSY

10

As indicated by the foregoing examples, the
present invention may be used to advantage to diagnose
cancer in presently asymptomatic patients. To
illustrate this point, a patient with a two year history

15   of high PSA levels (>12 μg/ml), had a needle biopsy of
the prostate performed two weeks prior to the analysis
set forth below. The biopsy did not reveal the presence
of malignancy. It is also noteworthy that a prior
biopsy performed 18 months earlier was also negative.

20   Before obtaining a 20 ml blood sample, the
patient was given a digital rectal exam and a gentle
massage of his enlarged prostate with the intention of
increasing the occurrence of tumor cells in the blood.
The blood sample was enriched using the methods of the

25   present invention. The enriched fraction was examined
by microscopy employing a Wrights-Giemsa stain.
Morphological examination of the isolated cells revealed
their malignant character. Clearly this patient had
cancer. Given the high PSA levels observed, a diagnosis

30   of prostate cancer is likely. The origin of the cells
may be determined using appropriate reagents as
described herein. The results presented in this example

reveal that the methods of the present invention can be used to detect cancers which might otherwise go undetected.

5   The notion of employing a localized massage to promote shedding of tumor cells into blood as a means of enhancing sensitivity of the blood test is a concept with considerable merit. Cells that are released into the circulation by this approach, following isolation may be used for a variety of different purposes. In the

10   case of cells isolated with ferrofluids, isolated cells can be readily cultured and/or cloned. The resultant cell lines can be used to assess a variety of malignant cell characteristics such as chemotherapeutic sensitivity and growth factor dependency.

15

EXAMPLE 9

Tests Kits for diagnosing various aspects of cancer.

Also contemplated for use in the present invention are test kits comprising the reagents used to

20   perform the assay of the invention. Such kits are designed for particular applications. Reagents may be assembled to facilitate screening of patients for circulating rare cells, including but not limited to tumor cells. In this embodiment, the kits contain

25   colloidal magnetic particles comprising a magnetic core material, a protein base coating material and a biospecific ligand which binds specifically to a characteristic determinant present on the cancer cell to be isolated. The kit also includes at least one

30   additional biospecific reagent which has affinity for a second characteristic determinant on the cancer cell to be isolated which differs from the determinant

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recognized by the biospecific ligand. The kit also includes a cell specific dye for excluding non-nucleated cells and other non-target sample components from analysis.

5   A typical kit according to this invention may include anti-EpCAM coupled directly or indirectly to magnetic nanoparticles, and a pair of monoclonal antibodies, the first antibody recognizing a cancer specific determinant and the second antibody having

10   affinity for a non-tumor cell determinant, e.g., a pan leukocyte antigen. The kit also contains a nucleic acid dye to exclude non-nucleated cells from analysis. The kit of the invention may optionally contain a biological buffer, a permeabilization buffer, a protocol,

15   separation vessels, analysis chamber, positive cells or appropriate beads and an information sheet.

        The kits described above may also be produced to facilitate diagnosis and characterization of particular cancer cells detected in circulation. In

20   this embodiment, the kits contain all of the items recited above, yet also preferably contain a panel of cancer specific monoclonal antibodies. Using breast cancer as an example, a kit for diagnosis may contain anti-MUC-1, anti-estrogen, anti-progesterone receptor

25   antibodies, anti-CA27.29, anti-CA15.3, anti-cathepsin D, anti-p53, anti-urokinase type plasminogen activator, anti-epidermal growth factor, anti-epidermal growth factor receptor, anti-BRCA1, anti-BRCA2, anti-prostate specific antigen, anti-plasminogen activator inhibitor,

30   anti-Her2-neu antibodies or a subset of the above.

        A kit is also provided for monitoring a patient for recurring disease and/or residual cells

following eradication of the tumor.   In this embodiment,
the type of cancer will already have been diagnosed.
Accordingly, the kit will contain all of the reagents
utilized for screening biological samples for cancer yet

5   also contain an additional antibody specific for the
type of cancer previously diagnosed in the patient.
Again using breast cancer as an example such a kit might
contain anti-MUC-1.   Alternatively, the kit may contain
anti-Her2-neu.

10   The kits of the invention may be customized
for screening, diagnosing or monitoring a variety of
different cancer types.   For example, if the kits were
to be utilized to detect prostate cancer, the antibodies
included in the kit would be specific for prostate

15   tissue.   Suitable antibodies or markers for this purpose
include anti-prostate specific antigen, free PSA,
prostatic acid phosphatase, creatine kinase, thymosin b-
15, p53, HPC1 basic prostate gene and prostate specific
membrane antigen.   If a patient were to be screened for

20   the presence of colon cancer, an antibody specific for
carcinoembryonic antigen (CEA) may be included in the
kit.   Kits utilized for screening patients with bladder
cancer may contain antibodies to nuclear matrix protein
(NMP22), Bard Bladder tumor antigen (BTA) or fibrin

25   degradation products (FDP).   Markers are known for many
different cancer types.

The cells isolated using the kits of the
invention may be further studied for morphology, RNA
associated with the organ of origin, surface and

30   intracellular proteins, especially those associated with
malignancy.   Based on existing information on such
molecules, it should be possible to determine from their

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expression on the isolated cell, the metastatic

potential of the tumor via analysis of the circulating

cells.

It is an object of the invention to provide

5   kits for any cancer for which specific markers are

known. A list summarizing those markers known at this

time and the usefulness and/or indication follows:

I   Indicative of tumor origin
        Muc-1 -- breast
10   PSA, PSMA -- prostate
        CEA -- colon
        CYPRA 21-1 -- lung
        CA 125 -- ovarian
        cytokeratins -- see list
15   anti-HI67
II Cell cycle
        nucleic acid dye
        cyclin A, C & E
        p27
20

III Cell viability/apoptosis
        Fas (CD95)
        amexin V
        anti-metalloproteinases
25

IV Drug sensitivity
        estrogen, progesterone & androgen receptors
        HER-2/neu

30   V.   Drug resistance
        P-glycoprotein (MDR)
        t-glutamylcysteine synthase
        taxol-resistance-associated-gene-1-5
        cis-diamminedichloroplatinum II resistance genes
35   thymidylate synthetase
        protein kinase C
        telomerase

VI. Staging
40   Lewis A
        C
        BRCA-1 BRCA-2
        CA15.3 (Muc-1),   CA 27.29,   CA 19.9
        LASA

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p53
cathepsin D
ras oncogene

The following table provideS different cytokeratin
markers that may be used to assess tissue origin of
cells isolated using the methods of the present
invention.

TABLE IX

CYTOKERATIN MARKERS

Cytokeratin Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Adrenal Cortex	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
Endometrium	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Esophagus	-	-	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	+	+	-
Gastro-Intestinal	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+
Kidney	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	+	-
Liver	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Lung Columnar	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+
Lung Basal	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-
Mammary Gland Luminal	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Mammary Gland Basal	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
Mesothelium	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Oral	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Ovary	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Pancreas	-	-	-	+	+	-	+	+	-	-	-	-	+	-	-	+	+	+	-
Pituitary Endocrine cells	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
Pituitary Follicular cells	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
Prostate Basal	-	-	-	-	+	+	-	+	-	+	-	-	+	+	-	-	+	+	+	-
Prostate Luminal	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
Skin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testis	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
Thymus	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+
Thyroid	-	-	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-	+	+	-
Urinary Bladder	-	-	-	+	+	-	+	+	-	-	-	-	+	-	-	-	-	+	-	+
Uterine Cervix	-	-	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	-
Non-Epithelial:																				
Mammary adenocarcinoma	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Prostate adenocarcinoma	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
Pancreatic adenocarcinoma	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+
Gastro-Intestinal adenocarcinoma	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+

Endometrium adenocarcinoma	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Lung adenocarcinoma	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Lung SCC	-	-	-	+	-	-	-	+	-	-	-	-	+	+	+	+	+	+	-	-
Liver	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-
Kidney renal cell tumor	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	+	-
Oral SCC	-	-	+	+	-	+	-	-	-	+	-	-	+	-	-	-	-	-	+	-
Ovary	-	-	-	+	+	-	+	+	-	+	-	-	+	-	-	-	-	+	+	-
Pituitary adenoma	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
Testis	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
Thyroid	-	-	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-	+	+	-
Urinary Bladder	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+
Uterine cervix	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+	+	+	-	
Valvular carcinoma	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-

The following demonstrates how the practice of the
methods of the invention is facilitated by means of a
kit for use in detection of circulating breast cancer
cells:

As described above, the kit starts with reagents,
devices and methodology for enriching tumor cells from
whole blood. The kit would contain reagents to test for
breast cancer cells in a blood sample which will assess
six factors or indicators. The analytical platform
needs to be configured such that the reporter molecules
DAPi, CY2, CY3, CY3.5, CY5, and CY5.5 will be
discriminated by the appropriate excitation and
emmission filters. The analytical platform in this
example uses a fluorescent microscope equipped with a
mercury arc lamp, and the appropriate filter sets for
assessing the wavelengths of the detection labels
employed. All of the markers are introduced at one time
with this method. DAPi, which is excited with UV light,
stains nucleic acids, and will be used to determine the
nuclear morphology of the cell. CAM 5.2 labelled with
CY2 will be used to stain the control cells. CY3

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labelled C11 will be used to label cytokeratins 7, 8, 18, and 19. An antibody conjugated with CY3.5 will be used to label HER-2/neu. An antibody conjugated with CY5 will be used to label Muc-1. An antibody conjugated to CY5.5 will be used to label estrogen receptors. By using the appropriate excitation and emmission filters, the cancer cells will be identified.

Examples of different types of cancer that may be detected using the compositions, methods and kits of the present invention include apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell and transitional cell reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing's sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, throphoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, leydig cell tumor, papilloma, sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma,

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neuroblastoma, neuroepithelioma, neurofibroma, neuroma,

paraganglioma, paraganglioma nonchromaffin,

antiokeratoma, angioma sclerosing, angiomatosis,

glomangioma, hemangioendothelioma, hemangioma,

5   hemangiopericytoma, hemangiosarcoma, lymphangioma,

lymphangiomyoma, lymphangiosarcoma, pinealoma,

carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes,

fibrosarcoma, hemangiosarcoma, leiomyosarcoma,

leukosarcoma, liposarcoma, lymphangiosarcoma,

10   myosarcoma, myxosarcoma, ovarian carcinoma,

rhabdomyosarcoma, sarcoma (Kaposi's, and mast-cell),

neoplasms (e.g., bone, digestive system, colorectal,

liver, pancreatic, pituitary, testicular, orbital, head

and neck, central nervous system, acoustic, pelvic,

15   respiratory tract, and urogenital), neurofibromatosis,

and cervical dysplasia.

The present invention is not limited to the

detection of circulating epithelial cells only.

Endothelial cells have been observed in the blood of

20   patients having a myocardial infarction. Endothelial

cells, myocardial cells, and virally infected cells,

like epithelial cells, have cell type specific

determinants recognized by available monoclonal

antibodies. Accordingly, the methods and the kits of

25   the invention may be adapted to detect such circulating

endothelial cells. Additionally, the invention allows

for the detection of bacterial cell load in the

peripheral blood of patients with infectious disease,

who may also be assessed using the compositions, methods

30   and kits of the invention.

Several citations to journal articles, US Patents

and US Patent applications are provided hereinabove.

The subject matter of each of the foregoing citations is incorporated by reference in the present specification as though set forth herein in full.

While certain of the preferred embodiments of the
5   present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the spirit of the present invention, the full scope of which is
10   delineated in the following claims.

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What is claimed is:

1. A method for detecting and enumerating rare cells in a mixed cell population, the presence of said rare cells in said population being indicative of a

5   disease state, comprising:

a) obtaining a biological specimen from a test subject, said specimen comprising a mixed cell population suspected of containing said rare cells;

b) preparing an immunomagnetic sample wherein

10   said biological specimen is mixed with magnetic particles coupled to a biospecific ligand which reacts specifically with the rare cells, to the substantial exclusion of other sample components;

c) contacting said immunomagnetic sample with

15   at least one biospecific reagent which labels said rare cells; and

d) analyzing said labeled rare cells to determine the presence and number of any rare cells in said immunomagnetic sample, the greater the number of

20   rare cells present in said sample the greater the severity of said disease state.

2. A method as claimed in claim 1, wherein as an intermediate step between the preparation of the

25   immunomagnetic sample and contacting said immunomagnetic sample with at least one biospecific reagent, said immunomagnetic sample is subjected to a magnetic field to produce a rare cell enriched cell suspension as the immunomagnetic sample.

30

3. A method as claimed in claim 3, wherein the volume of said immunomagnetic sample containing said

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enriched rare cells is reduced.

4.   A method as claimed in claim 1, wherein before
analysis said immunomagnetic sample is separated into a
labeled rare cell-containing fraction and an unlabeled
fraction.

5.   A method as claimed in claim 4, wherein said
magnetic particles are colloidal and separation is
effected by subjecting said immunomagnetic sample to a
magnetic gradient field.

6.   A method as claimed in claim 1, wherein said
rare cell is selected from the group consisting of
endothelial cells, fetal cells in maternal circulation,
bacterial cells, myocardial cells, epithelial cells, and
virally infected cells.

7.   A method for detecting and enumerating cancer
cells in a mixed cell population, comprising:
        a) obtaining a biological specimen from a
test subject, said specimen comprising a mixed cell
population suspected of containing said cancer cells;
        b)   preparing an immunomagnetic sample
wherein said biological specimen is mixed with magnetic
particles coupled to a biospecific ligand which reacts
specifically with the cancer cells, to the substantial
exclusion of other sample components;
        c) contacting said immunomagnetic sample
with at least one biospecific reagent which labels said
cancer cells; and
        d) analyzing said labeled cancer cells to

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determine the presence and number of any cancer cells in
said immunomagnetic sample, the greater the number of
cancer cells present in said sample the greater the
severity of said cancer.

5

    8.   A method as claimed in claim 7 wherein
said magnetic particles are colloidal and before
analysis said immunomagnetic sample is separated into a
labelled cancer cell-containing fraction and an
10   unlabelled fraction.

    9.   A method as claimed in claim 7, wherein
said colloidal magnetic particles and said at least one
biospecific reagent are sequentially mixed with said
15   biological specimen, and, as an intermediate step, said
immunomagnetic sample is subjected to a magnetic field
to produce a cancer cell enriched cell suspension as the
immunomagnetic sample.

20   10.   A method as claimed in claim 9 wherein
before analysis said immunomagnetic sample is separated
into a labelled cancer cell-containing fraction and an
unlabelled fraction.

25   11.   A method as claimed in claim 10, wherein
said labelled cancer cell-containing fraction is
analyzed by a process selected from the group consisting
of multiparameter flow cytometry, immunofluorescent
microscopy, laser scanning cytometry, bright field base
30   image analysis, capillary volumetry, spectral imaging
analysis manual cell analysis and automated cell
analysis.

12. A method as claimed in claim 8, wherein said biospecific ligand is a monoclonal antibody specific for at least one cancer cell determinant, and said at least one biospecific reagent comprises at least one additional monoclonal antibody specific for a second cancer cell determinant and a third monoclonal antibody specific for an antigen present on a non tumor-cell, and said method further comprises adding to said labeled cancer cell-containing fraction a cell specific dye to allow exclusion of residual non-nucleated cells and cell debris from analysis.

13. A method as claimed in claim 12, wherein said method is applied to detect and enumerate residual cancer cells in said biological specimen following at least one tumor eradication procedure.

14. A method as claimed in claim 12, wherein said method further comprises the step of assessing the malignant status of said separated cancer cells by immunocytochemical analysis.

15. A method as claimed in claim 12, wherein said biological specimen is obtained from said test subject periodically and assessed for the presence and number of circulating cancer cells as an indicator of progression of said disease state.

16. A method as claimed in claim 12, wherein said biological specimen is obtained from said test subject periodically to assess for the presence and number of circulating cancer cells as an indicator of

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said patient's response to cancer eradication
procedures.

17.   A method as claimed in claim 12, wherein
said biospecific ligand binds specifically to an
epithelial cell adhesion molecule.

18.   A method as claimed in claim 12, wherein
said one or more biospecific reagent binds specifically
to an intracellular cytokeratin.

19.   A method as claimed in claim 12, wherein
said one or more biospecific reagent is specific for a
cytokeratin selected from the group consisting of
cytokeratin 7, 8, 18, and 19.

20.   A method as claimed in claim 12, wherein
said biospecific ligand has binding affinity for
epithelial cell adhesion molecule, and said at least one
biospecific reagent has affinity for an intracellular
cytokeratin selected from the group consisting of
cytokeratin 7, 8, 18 and 19.

21.   A method as claimed in claim 12, wherein
said patient has been diagnosed with a cancer selected
from the group consisting of prostate cancer, breast
cancer, colon cancer apudoma, choristoma, branchioma,
malignant carcinoid syndrome, carcinoid heart disease,
carcinoma e.g., Walker, basal cell, basosquamous, Brown-
Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, merkel
cell, mucinous, non-small cell lung, oat cell,
papillary, scirrhous, bronchiolar, bronchogenic,

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squamous cell and transitional cell

reticuloendotheliosis, melanoma, chondroblastoma,

chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant

cell tumors, histiocytoma, lipoma, liposarcoma,

5   mesothelioma, myxoma, myxosarcoma, osteoma,

osteosarcoma, Ewing's sarcoma, synovioma, adenofibroma,

adenolymphoma, carcinosarcoma, chordoma, mesenchymoma,

mesonephroma, myosarcoma, ameloblastoma, cementoma,

odontoma, teratoma, throphoblastic tumor,

10   adenocarcinoma, adenoma, cholangioma, cholesteatoma,

cylindroma, cystadenocarcinoma, cystadenoma, granulosa

cell tumor, gynandroblastoma, hepatoma, hidradenoma,

islet cell tumor, leydig cell tumor, papilloma, sertoli

cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma,

15   myoblastoma, myoma, myosarcoma, rhabdomyoma,

rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma,

medulloblastoma, meningioma, neurilemmoma,

neuroblastoma, neuroepithelioma, neurofibroma, neuroma,

paraganglioma, paraganglioma nonchromaffin,

20   antiokeratoma, angioma sclerosing, angiomatosis,

glomangioma, hemangioendothelioma, hemangioma,

hemangiopericytoma, hemangiosarcoma, lymphangioma,

lymphangiomyoma, lymphangiosarcoma, pinealoma,

carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes,

25   fibrosarcoma, hemangiosarcoma, leiomyosarcoma,

leukosarcoma, liposarcoma, lymphangiosarcoma,

myosarcoma, myxosarcoma, ovarian carcinoma,

rhabdomyosarcoma, sarcoma (Kaposi's, and mast-cell),

neoplasms (e.g., bone, digestive system, liver,

30   pancreatic, pituitary, testicular, orbital, head and

neck, central nervous system, acoustic, pelvic,

respiratory tract, and urogenital), neurofibromatosis,

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and cervical dysplasia.

22. A method of diagnosing early stage cancer in a
test subject, comprising:
5   a) obtaining a biological specimen from a test
subject, said specimen comprising a mixed cell
population suspected of containing cancer cells;
             b) preparing a cell fraction from said
biological specimen, said cell fraction being enriched
10   for cancer cells, if present in said biological
specimen; and
             c) analyzing said enriched fraction for the
presence of said cancer cells; the presence of said
cancer cells in said specimen indicating the presence of
15   early stage cancer in said test subject.

23. A method as claimed in claim 22, wherein
preparation of said immunomagnetic cell fraction
comprises mixing said biological specimen with magnetic
20   particles coupled to a biospecific ligand which binds
specifically to said cancer cells, to the substantial
exclusion of other biological specimen components and
subjecting the specimen-magnetic particle mixture to a
magnetic field to produce a cell suspension enriched in
25   magnetic particle-bound cancer cells as an
immunomagnetic sample.

24. A method as claimed in claim 23, wherein
before analysis said immunomagnetic sample is contacted
30   with at least one biospecific reagent that labels said
cancer cells.

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25.   A method as claimed in claim 23, wherein the
volume of said immunomagnetic sample containing said
enriched magnetic particle-bound cancer cells is
reduced.

5

26.   A method as claimed in claim 23, wherein said
immunomagnetic sample is further separated into a
labeled magnetic fraction and an unlabeled fraction by
subjecting said immunomagnetic sample to a magnetic
10   gradient field.

27.   A method as claimed in claim 23, wherein said
magnetic particles are colloidal nanoparticles.

15   28.   A coated, magnetic particle comprising a
nanoparticle core of magnetic material, and a base
coating material on said magnetic core in an amount
sufficient to hinder non-specific binding of biological
macromolecules to said magnetic core.

20

29.   A coated, magnetic particle comprising a
nanoparticle core of magnetic material, a base coating
material that forms a discontinous coating on said
magnetic core, providing at least one area of
25   discontinuity which, if accessible, contributes to non-
specific binding of said base coated particle to
biological macromolecules, and an additional coating
material that hinders access to said at least one area
of discontinuity by said biological macromolecules.

30

30.   A magnetic particle as claimed in claim 28,

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wherein said magnetic core material comprises at least
one transition metal oxide and said base coating
material comprises a protein.

5   31.   A magnetic particle as claimed in claim 30,
wherein said additional coating material is one member
of a specific binding pair which is coupled to said base
coating material.

10   32.   A magnetic particle as claimed in claim 31,
wherein said one member of said specific binding pair is
coupled to said base coating material through a
bifunctional linking compound.

15   33.   A magnetic particle as claimed in claim 31,
wherein said specific binding pair is selected from the
group consisting of biotin-streptavidin, antigen-
antibody, receptor-hormone, receptor-ligand, agonist-
antagonist, lectin-carbohydrate, Protein A-antibody Fc,
20 and avidin-biotin.

         34.   A magnetic particle as claimed in claim 31,
wherein said specific binding pair is biotin-
streptavidin and streptavidin is coupled to said base
25 coating material.

         35.   A magnetic particle as claimed in claim 34,
wherein a fraction of the streptavidin which is coupled
to said base coating material is bound to biotinylated
30 antibody and the remainder of said streptavidin is
unbound.

36. A magnetic particle as claimed in claim 34, wherein a fraction of the streptavidin which is coupled to said base coating material is bound to biotinylated antibody and a substantial portion of said streptavidin

5   remaining is bound to free biotin.

37. A magnetic particle as claimed in claim 34, wherein a fraction of the streptavidin which is coupled to said base coating material is bound to biotinylated

10   antibody and a substantial portion of said streptavidin remaining is bound to a biotin-protein conjugate.

38. A magnetic particle as claimed in claim 34, wherein the protein component of said biotin-protein

15   conjugate comprises bovine serum albumin.

39. A coated magnetic particle, as claimed in claim 29, having between 70-90% magnetic mass.

20   40. A coated magnetic particle, as claimed in claim 29, wherein a major portion of said particles have a particle size in the range of 90-120 nm.

41. A coated magnetic particle, as claimed in

25   claim 29, wherein a major portion of said particles have a particle size in the range of 120-150 nm.

42. A coated magnetic particle, as claimed in claim 29, wherein a major portion of said particles have

30   a particle size in the range of 90-150 nm.

43. A composition comprising a plurality of

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magnetic particles as claimed in claim 29, suspended in a biologically compatible aqueous medium.

44.   A coated magnetic particle comprising a nanoparticle core material of a magnetic transition metal oxide, a protein base coating material and an additional coating material coupled to said base coating material through a biofunctional linking compound, said additional coating material being one member of a specific binding pair selected from the group consisting of biotin-streptavidin, antigen-antibody, receptor-hormone, receptor-ligand, agonist-antagonist, lectin-carbohydrate, Protein A-antibody Fc, and avidin-biotin.

45.   A coated magnetic particle as claimed in claim 44, wherein said core material is magnetite, said base coating material is bovine serum albumin, said specific binding pair is biotin-streptavidin and streptavidin is coupled to said base coating material.

46.   A coated magnetic particle as claimed in claim 45, wherein a fraction of the streptavidin which is coupled to said base coating material is bound to biotinylated antibody and a substantial portion of said streptavidin remaining is bound to a biotin-bovine serum albumin conjugate.

47.   A composition comprising a plurality of magnetic particles as claimed in claim 46, suspended in a biologically compatible aqueous medium.

48.   A test kit for screening a patient sample for

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the presence of circulating rare cells comprising:

a) coated magnetic nanoparticles comprising a magnetic core material, a protein base coating material, and an antibody that binds specifically to a

5   first characteristic determinant of said rare cell, said antibody being coupled, directly or indirectly, to said base coating material;

b) at least one antibody having binding specificity for a second characteristic determinant of

10   said rare cell; and

c) a cell specific dye for excluding sample components other than said rare cells from analysis.

49. A kit as claimed in claim 48, said kit further

15   containing an antibody which has binding affinity for non-target cells, a biological buffer, a permeabilization buffer, a protocol and optionally, an information sheet.

20   50. A kit as claimed in claim 48, wherein said rare cells are selected from the group consisting of endothelial cells, fetal cells in maternal circulation, bacterial cells, myocardial cells, epithelial cells, and virally infected cells.

25

51. A kit for screening a patient sample for the presence of circulating tumor cells, comprising:

a) coated magnetic nanoparticles comprising a magnetic core material, a protein base coating material,

30   and anti-EpCAM coupled, directly or indirectly, to said base coating material;

b) at least one antibody having binding

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specificity for a cancer cell determinant; and

         c)   cell specific dye for excluding sample components other than said tumor cells from analysis.

5   52.   A kit as claimed in claim 51, said kit further containing an antibody which has binding affinity for non-tumor cells, a biological buffer, a permeabilization buffer, a protocol and optionally, an information sheet.

10

       53.   A kit as claimed in claim 51 for screening patients for breast cancer, wherein said at least one antibody having binding specificity for a cancer cell determinant specifically binds a breast cancer cell

15   determinant, said determinant being selected from the group of determinants consisting of MUC-1, estrogen, progesterone receptor, cathepsin D, p53, urokinase type plasminogen activator, epidermal growth factor, epidermal growth factor receptor, BRCA1, BRCA2, CA27.29,

20   CA15.5, prostate specific antigen, plasminogen activator inhibitor and Her2-neu.

       54.   A kit as claimed in claim 51 for screening patients for prostate cancer, wherein said at least one

25   antibody having binding specificity for a cancer cell determinant specifically binds a prostate cancer cell determinant, said determinant being selected from the group of determinants consisting of prostate specific antigen, prostatic acid phosphatase, thymosin b-15, p53,

30   HPC1 basic prostate gene, creatine kinase and prostate specific membrane antigen.

55. A kit as claimed in claim 51 for screening patients for colon cancer, wherein said at least one antibody having binding specificity for a cancer cell determinant specifically binds a colon cancer cell

5   determinant, said determinant being selected from the group of determinants consisting of carcinoembryonic antigen, C protein, APC gene, p53 and matrix metalloproteinase (MMP-9).

10   56. A kit as claimed in claim 51 for screening patients with bladder cancer, wherein said at least one antibody having binding specificity for a cancer cell determinant specifically binds a bladder cancer cell determinant, said determinant being selected from the

15   group of determinants consisting of nuclear matrix protein (NMP22),Bard Bladder tumor antigen (BTA), and fibrin degradation product (FDP).

57. A test kit as claimed in claim 51, wherein

20   said at least one antibody comprises a panel of antibodies each having binding specificity for a different cancer cell characteristic determinant.

58. A kit for screening a patient sample for the

25   presence of circulating breast cancer cells, comprising:
        a) coated magnetic nanoparticles comprising a magnetic core material, a protein base coating material, and anti-EpCAM coupled, directly or indirectly, to said base coating material;

30   b) at least one antibody having binding specificity for a breast cancer cell determinant; and

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c)   cell specific dye for excluding sample components other than said breast cancer cells from analysis.

5   59.   A kit for screening a patient sample for the presence of circulating prostate cancer cells, comprising:

a) coated magnetic nanoparticles comprising a magnetic core material, a protein base coating material,

10   and anti-EpCAM coupled, directly or indirectly, to said base coating material;

b)   at least one antibody having binding specificity for a prostate cancer cell determinant; and

15   c)   cell specific dye for excluding sample components other than said prostate cancer cells from analysis.

60.   A kit for screening a patient sample for the

20   presence of circulating colon cancer cells, comprising:

a) coated magnetic nanoparticles comprising a magnetic core material, a protein base coating material, and anti-EpCAM coupled, directly or indirectly, to said base coating material;

25   b)   at least one antibody having binding specificity for a colon cancer cell determinant; and

c)   cell specific dye for excluding sample components other than said colon cancer cells from

30   analysis.

61.   A kit for screening a patient sample for the

presence of circulating bladder cancer cells,
comprising:

a) coated magnetic nanoparticles comprising a
magnetic core material, a protein base coating material,
and anti-EpCAM coupled, directly or indirectly, to said
base coating material;

b) at least one antibody having binding
specificity for a bladder cancer cell determinant; and

c) cell specific dye for excluding sample
components other than said bladder cancer cells from
analysis.

62. A test kit for monitoring a patient for the
recurrence of cancer, said test kit comprising:

a)   coated magnetic nanoparticles comprising a
magnetic core material, a protein base coating material,
and anti-EpCAM coupled, directly or indirectly, to said
base coating material;

b)   an antibody having binding specificity
for an intracellular cytokeratin marker;

c)   an antibody having binding specificity
for a predetermined cancer specific determinant which is
different from said intracellular cytokeratin marker;
and

d)   a cell specific dye for excluding sample
components other than said cancer cells from analysis.

63. A kit as claimed in claim 62, said kit further
containing an antibody having specificity for non-tumor
cells, a biological buffer, a permeabilization buffer, a
protocol and optionally, an information sheet.

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64. A kit as claimed in claim 62 for monitoring a patient with breast cancer, wherein said antibody having binding specificity for a predetermined cancer specific determinant binds specifically to a breast cancer cell determinant, said determinant being selected from the group of determinants consisting of MUC-1, estrogen, progesterone receptor, cathepsin D, p53, urokinase type plasminogen activator, epidermal growth factor, epidermal growth factor receptor, BRCA1, BRCA2, CA27.29, CA15.5, prostate specific antigen, plasminogen activator inhibitor and Her2-neu.

65. A kit as claimed in claim 62 for monitoring patients with prostate cancer, wherein said antibody having binding specificity for a predetermined cancer specific determinant binds specifically to a prostate cancer cell determinant, said determinant being selected from the group of determinants consisting of prostate specific antigen, prostatic acid phosphatase, thymosin b-15, p53, HPC1 basic prostate gene, creatine kinase and prostate specific membrane antigen.

66. A kit as claimed in claim 62 for monitoring patients with colon cancer, wherein said antibody having binding specificity for a predetermined cancer specific determinant binds specifically to a colon cancer cell determinant, said determinant being selected from the group of determinants consisting of carcinoembryonic antigen, Cand protein, APC gene, p53 and matrix metalloproteinase (MMP-9).

67. A kit as claimed in claim 62 for monitoring

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patients with bladder cancer, wherein said antibody having binding specificity for a predetermined cancer specific determinant binds to a bladder cancer cell determinant, said determinant being selected from the

5   group of determinants consisting of nuclear matrix protein (NMP22), Bard Bladder tumor antigen (BTA) and fibrin degradation product (FDP).

10   68.   A method for determining likelihood of cancer recurrence in a human subject previously treated for cancer,
comprising:
        a)   obtaining a blood sample from said
15   subject;
        b)   determining the number of epithelial cells in said sample;
        c)   comparing the number of epithelial cells with a statistically determined number of epithelial
20   cells from a group of tumor-free patient controls; and
        d)   assigning a likelihood of cancer recurrence when the number of epithelial cells exceeds a pre-determined value based on statistical averages of circulating epithelial cells from healthy subjects
25   compared with statistical averages of circulating epithelial cells from cancer patients.

        69.   The method of claim 68 further comprising
30   identifying the tissue origin of the epithelial cells.

        70.   The method of claim 69 wherein tissue origin

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of the epithelial cells is identified by cell surface
binding with selected tumor specific antibodies.

71. The method of claim 68 wherein tumor
recurrence is determined to be likely when epithelial
cell number in blood is about 5 to about 15 epithelial
cells per 20 ml of blood.

72. A method of distinguishing an organ confined
carcinoma from a carcinoma with metastatic properties,
comprising:

a) obtaining a blood sample from a test
subject;

b) determining the number of circulating
epithelial cells in said sample;

c) identifying the tissue origin of the
epithelial cells;

d) comparing the number of circulating tissue
specific epithelial cells determined for said test
subject with
a statistically determined average number of circulating
tissue specific epithelial cells from a group of healthy
subjects, a
group of organ-confined carcinoma patients and a group
of patients with metastatic carcinoma; and

e) identifying said human test subject cells
as metastatic when the number is within the
statistically determined average for the group of
patients with metastatic carcinoma.

73. The method of claim 72 wherein the carcinoma
is a carcinoma selected from the group consisting of
prostate cancer, breast cancer, colon cancer apudoma,
choristoma, branchioma, malignant carcinoid syndrome,

5   carcinoid heart disease, carcinoma e.g., Walker, basal
cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor,
in situ, Krebs 2, merkel cell, mucinous, non-small cell
lung, oat cell, papillary, scirrhous, bronchiolar,
bronchogenic, squamous cell and transitional cell

10   reticuloendotheliosis, melanoma, chondroblastoma,
chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant
cell tumors, histiocytoma, lipoma, liposarcoma,
mesothelioma, myxoma, myxosarcoma, osteoma,
osteosarcoma, Ewing's sarcoma, synovioma, adenofibroma,

15   adenolymphoma, carcinosarcoma, chordoma, mesenchymoma,
mesonephroma, myosarcoma, ameloblastoma, cementoma,
odontoma, teratoma, throphoblastic tumor,
adenocarcinoma, adenoma, cholangioma, cholesteatoma,
cylindroma, cystadenocarcinoma, cystadenoma, granulosa

20   cell tumor, gynandroblastoma, hepatoma, hidradenoma,
islet cell tumor, leydig cell tumor, papilloma, sertoli
cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma,
myoblastoma, myoma, myosarcoma, rhabdomyoma,
rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma,

25   medulloblastoma, meningioma, neurilemmoma,
neuroblastoma, neuroepithelioma, neurofibroma, neuroma,
paraganglioma, paraganglioma nonchromaffin,
antiokeratoma, angioma sclerosing, angiomatosis,
glomangioma, hemangioendothelioma, hemangioma,

30   hemangiopericytoma, hemangiosarcoma, lymphangioma,
lymphangiomyoma, lymphangiosarcoma, pinealoma,
carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes,

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fibrosarcoma, hemangiosarcoma, leiomyosarcoma,
leukosarcoma, liposarcoma, lymphangiosarcoma,
myosarcoma, myxosarcoma, ovarian carcinoma,
rhabdomyosarcoma, sarcoma (Kaposi's, and mast-cell),
5   neoplasms (e.g., bone, digestive system, liver,
pancreatic, pituitary, testicular, orbital, head and
neck, central nervous system, acoustic, pelvic,
respiratory tract, and urogenital), neurofibromatosis,
and cervical dysplasia.
10

74.   A method as claimed in claim 72 wherein the
carcinoma is a breast carcinoma.

15   75.   A method as claimed in claim 74 wherein a
number of epithelial cells in the human test subject's
blood greater than the average number in a group of
organ-confined breast carcinoma patients is indicative
of metastatic breast carcinoma.
20

76.   A method as claimed in claim 74 wherein a
number of epithelial cells in the human test subject's
blood which is higher than average number in a group of
healthy subjects but less than the average number in a
25   group of metastatic breast carcinoma patients is
indicative of organ-confined breast carcinoma.

77.   A method of following remission status in a
human cancer patient undergoing cancer therapy
30   treatment, comprising:
        a)   obtaining a blood sample from a human
cancer patient;

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b)   determining the number of circulating
epithelial cells in said sample prior to and
periodically subsequent to said cancer therapy treatment
wherein an increase over time in the number of
5   epithelial cells following treatment compared to the
number of epithelial cells just after treatment is
indicative of relapse and wherein maintenance of
epithelial cell levels comparable to levels just after
treatment is indicative of continuing remission.
10

78.   The method of claim 77 wherein the cancer
is selected from the group of cancers consisting of
prostate cancer, breast cancer, colon cancer apudoma,
choristoma, branchioma, malignant carcinoid syndrome,
15   carcinoid heart disease, carcinoma e.g., Walker, basal
cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor,
in situ, Krebs 2, merkel cell, mucinous, non-small cell
lung, oat cell, papillary, scirrhous, bronchiolar,
bronchogenic, squamous cell and transitional cell
20   reticuloendotheliosis, melanoma, chondroblastoma,
chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant
cell tumors, histiocytoma, lipoma, liposarcoma,
mesothelioma, myxoma, myxosarcoma, osteoma,
osteosarcoma, Ewing's sarcoma, synovioma, adenofibroma,
25   adenolymphoma, carcinosarcoma, chordoma, mesenchymoma,
mesonephroma, myosarcoma, ameloblastoma, cementoma,
odontoma, teratoma, throphoblastic tumor,
adenocarcinoma, adenoma, cholangioma, cholesteatoma,
cylindroma, cystadenocarcinoma, cystadenoma, granulosa
30   cell tumor, gynandroblastoma, hepatoma, hidradenoma,
islet cell tumor, leydig cell tumor, papilloma, sertoli
cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma,

myoblastoma, myoma, myosarcoma, rhabdomyoma,
rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma,
medulloblastoma, meningioma, neurilemmoma,
neuroblastoma, neuroepithelioma, neurofibroma, neuroma,
paraganglioma, paraganglioma nonchromaffin,
antiokeratoma, angioma sclerosing, angiomatosis,
glomangioma, hemangioendothelioma, hemangioma,
hemangiopericytoma, hemangiosarcoma, lymphangioma,
lymphangiomyoma, lymphangiosarcoma, pinealoma,
carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes,
fibrosarcoma, hemangiosarcoma, leiomyosarcoma,
leukosarcoma, liposarcoma, lymphangiosarcoma,
myosarcoma, myxosarcoma, ovarian carcinoma,
rhabdomyosarcoma, sarcoma (Kaposi's, and mast-cell),
neoplasms (e.g., bone, digestive system, liver,
pancreatic, pituitary, testicular, orbital, head and
neck, central nervous system, acoustic, pelvic,
respiratory tract, and urogenital), neurofibromatosis,
and cervical dysplasia.

79. A fraction of peripheral blood enriched for
circulating neoplastic cells, said enrichment being at
least 2,500 fold with reference to the sample of
peripheral blood from which said fraction was obtained.

80. A fraction of peripheral blood enriched for
circulating neoplastic cells, said enrichment being at
least 5,000 fold with reference to the sample of
peripheral blood from which said fraction was obtained.

81. A fraction of peripheral blood enriched for
circulating neoplastic cells, said enrichment being at

least 10,000 fold with reference to the sample of peripheral blood from which said fraction was obtained.

82.   A method as claimed in claim 8, wherein said
5   biological specimen is peripheral blood.

83.   A method for increasing numbers of circulating epithelial cells in a blood sample, comprising massaging a tissue suspected of being cancerous prior to obtaining
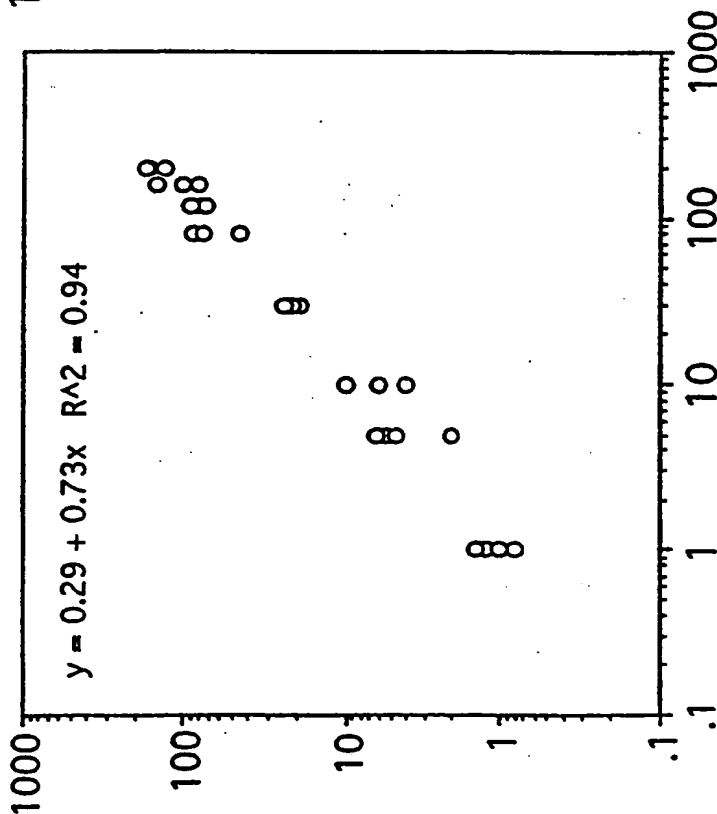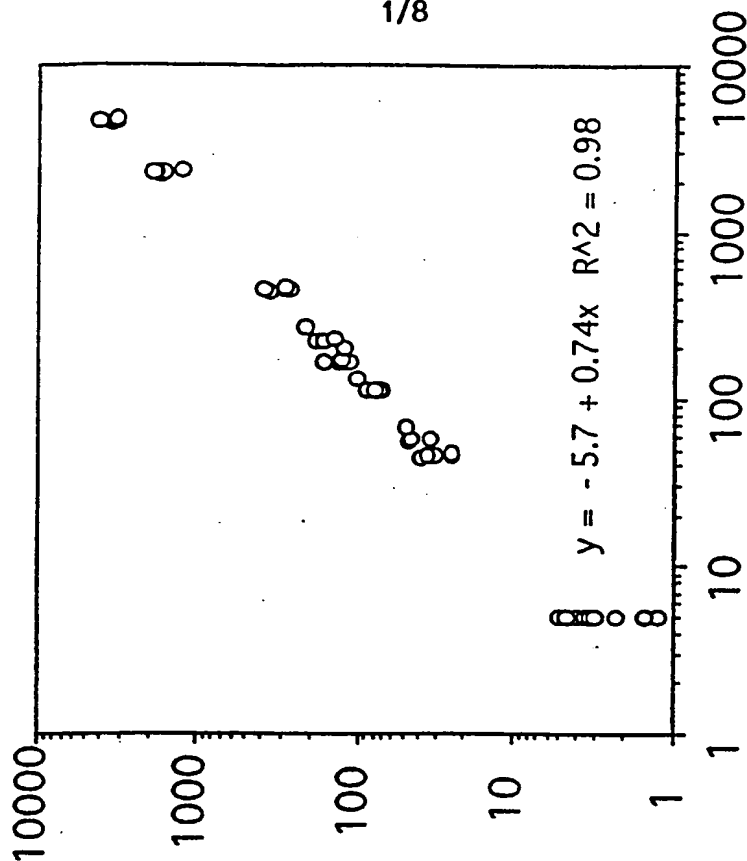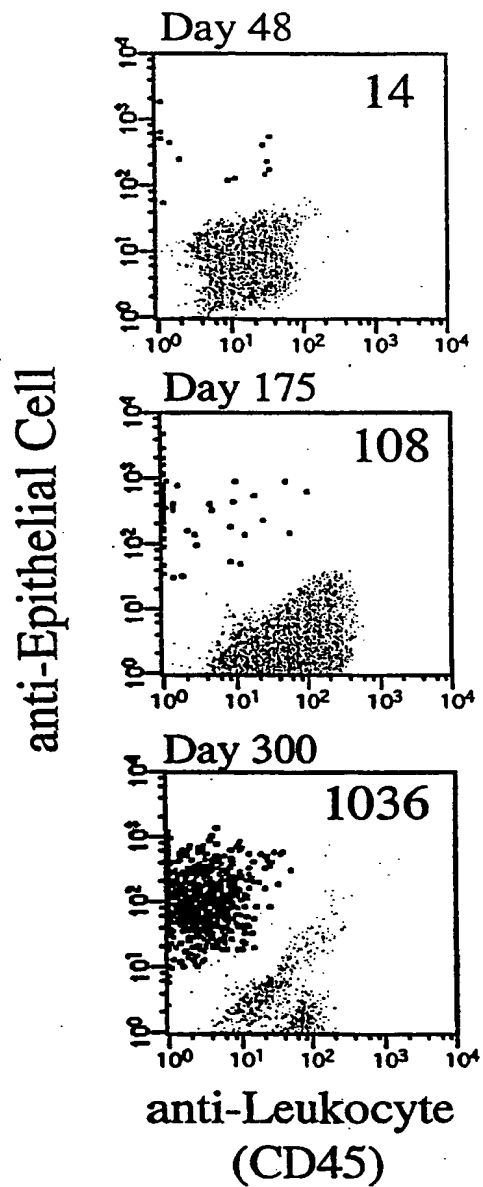10   said blood sample.

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Fig. 1B

y = - 5.7 + 0.74x   R^2 = 0.98

Fig. 1A

y = 0.29 + 0.73x   R^2 = 0.94

Tumor Cells Detected

Tumor Cells Spiked in 5 ml Blood

Figure 2
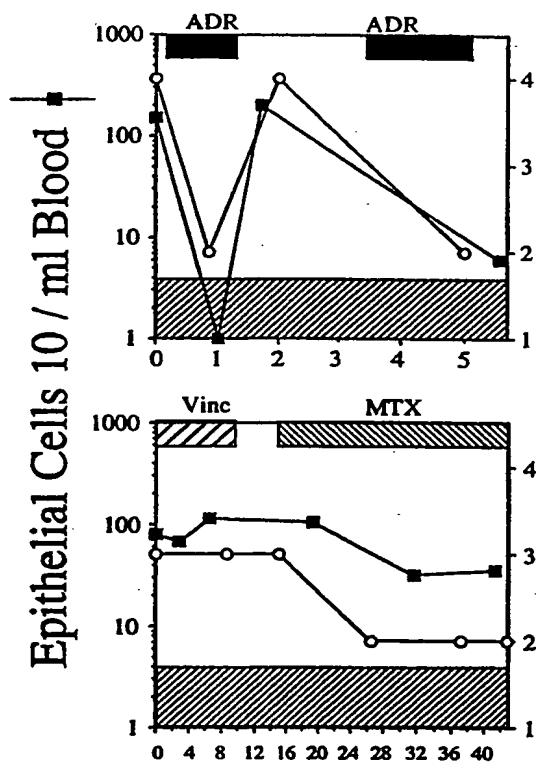
Fig. 3A   Fig. 3B
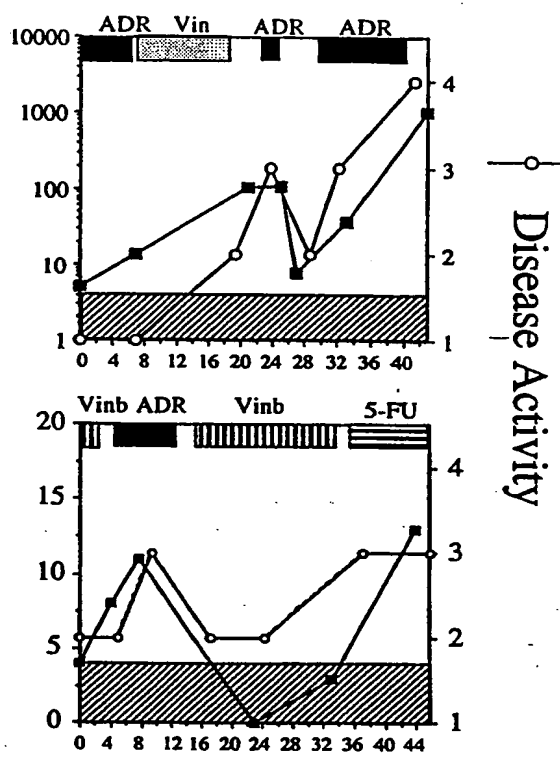
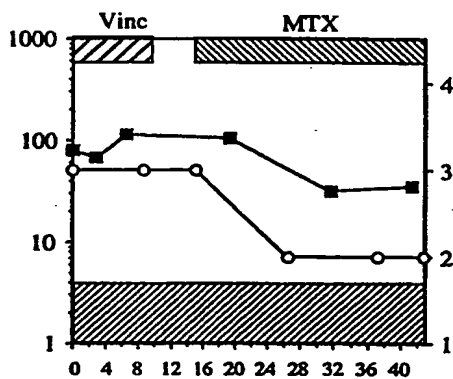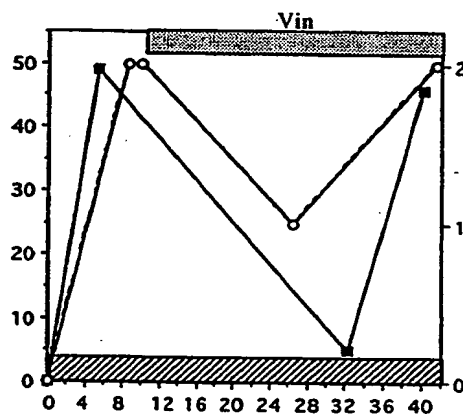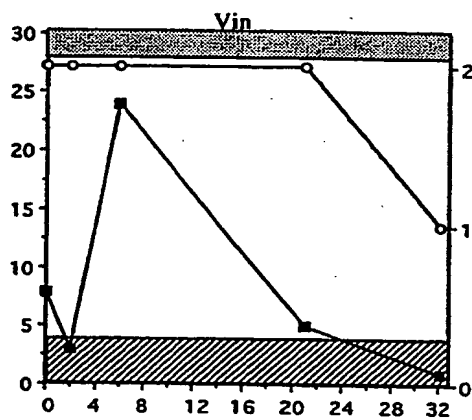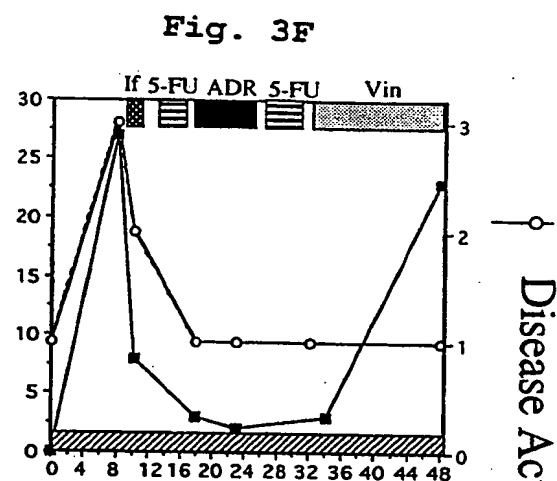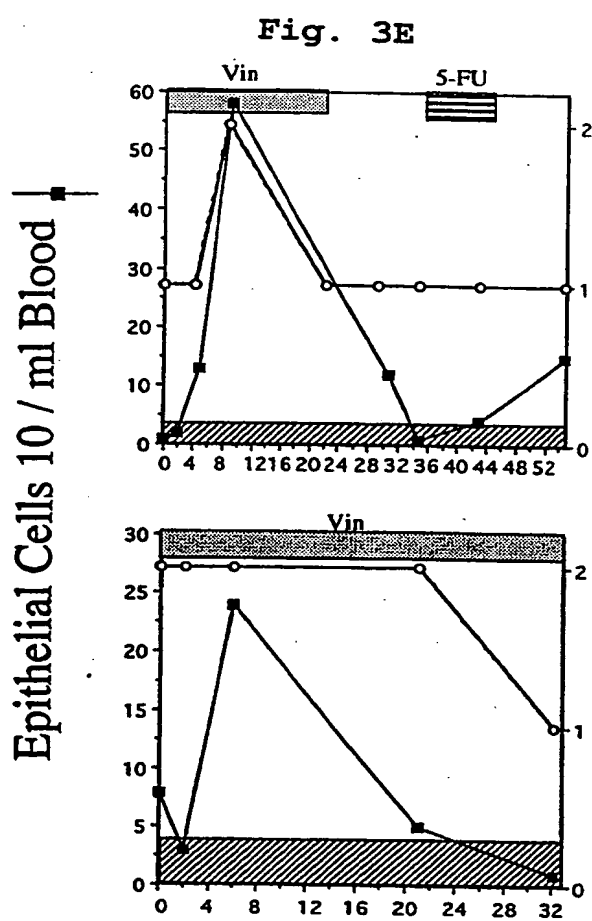Fig. 3C   Fig. 3D

Time (Weeks) -->

Fig. 3E

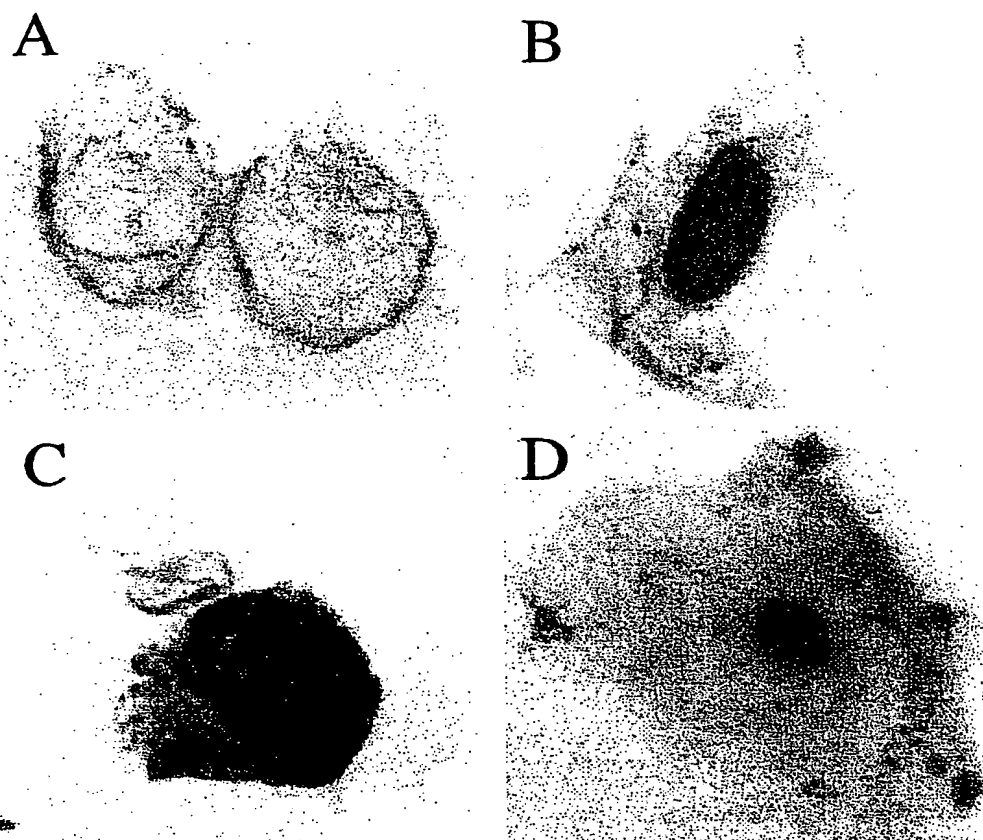Fig. 3F
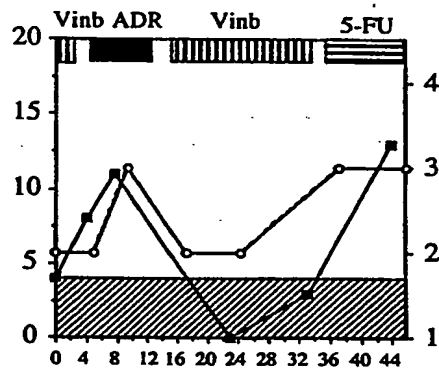
Fig. 3G   Time (Weeks) -->   Fig. 3H
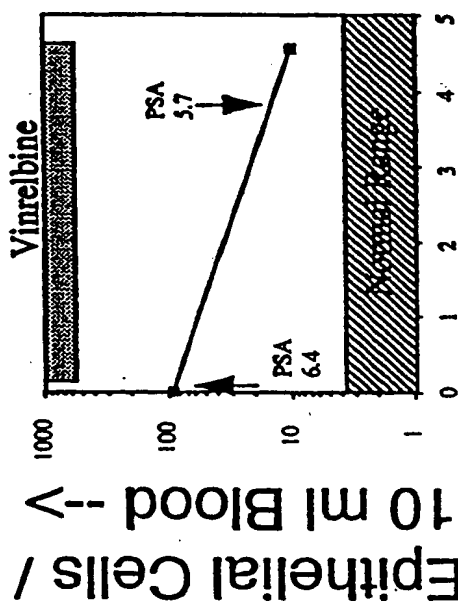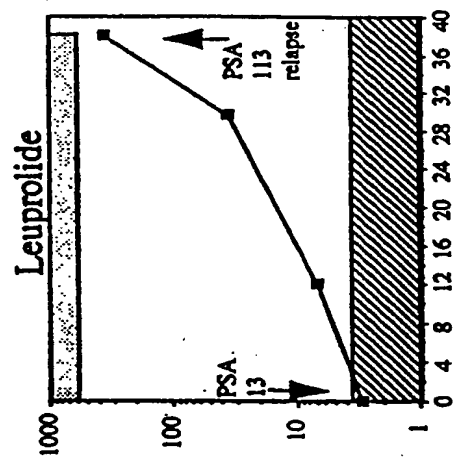
Figure 4

Fig. 5A

Fig. 5B

Fig. 5C
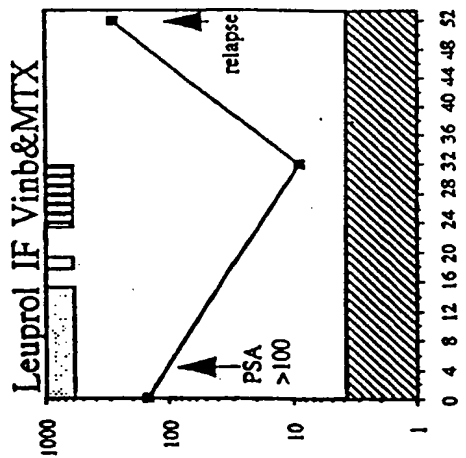
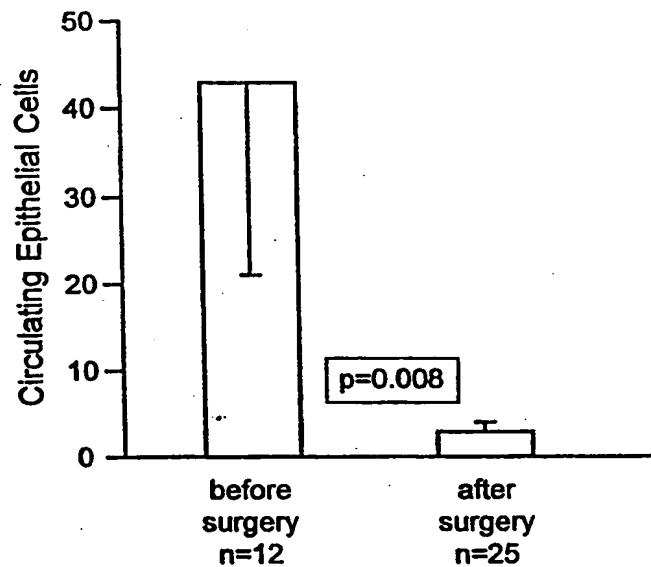# Colon Cancer Without Metastases
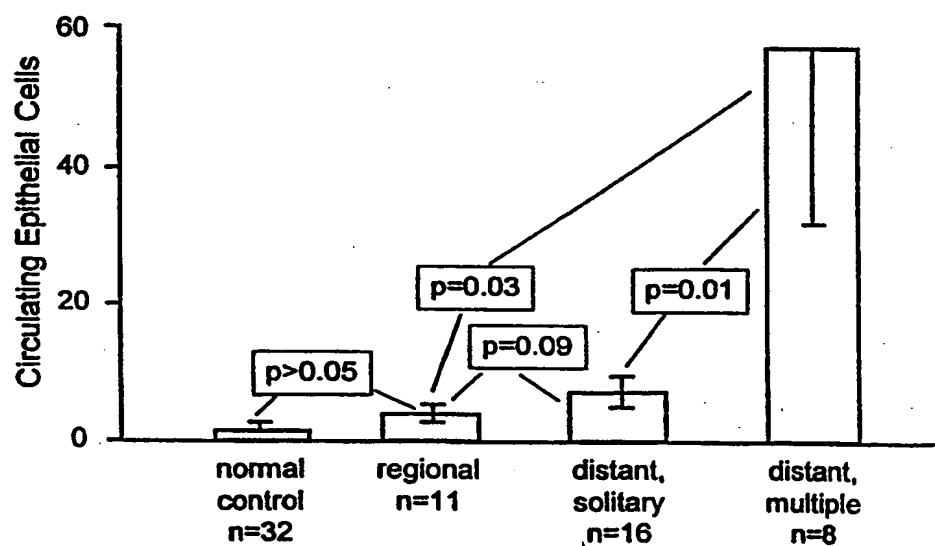

Figure 6

Metastatic Colon Cancer

Figure 7
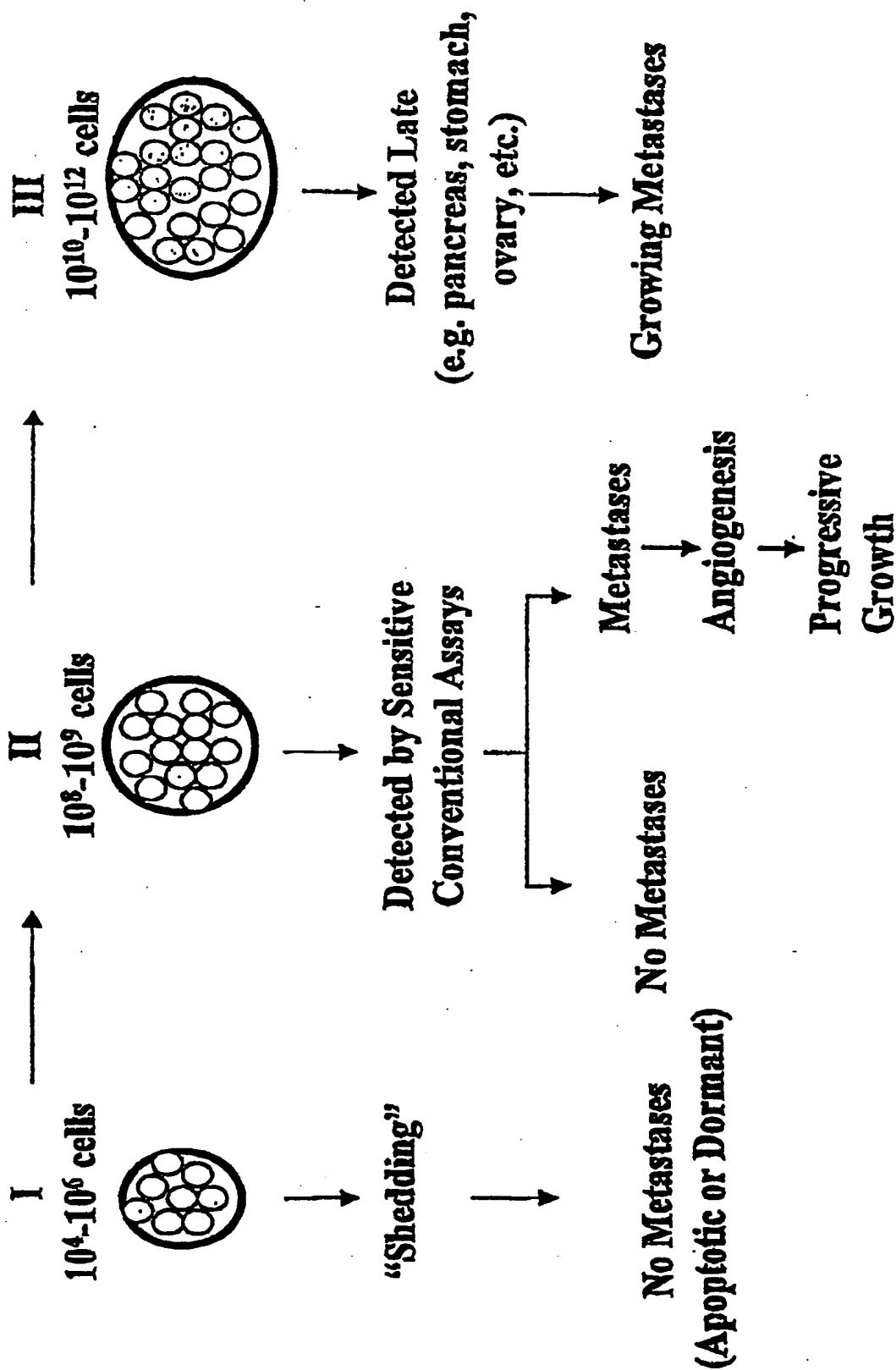
Figure 8

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

G 01 N 33/58, G 01 N 33/53, G 01 N 33/49, C 12 Q 1/06,
C 12 Q 1/24

According to International Patent Classification (IPC) or to both national classification and IPC: 6

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G 01 N, C 12 Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BEROIS, N. et al. Detection of Rare Human Breast Cancer Cells. Comparison of an Immunomagnetic Separation Method with Immunocytochemistry and RT-PCR. Anticancer Research, July-August 1997, Vol. 17, No. 4A, pages 2639-2646.	1-27
A	MAKAROVSKIY, A.N. et al. Application of Immunomagnetic Beads in Combination with RT-PCR for the Detection of Circulating Prostate Cancer Cells. Journal of Clinical Laboratory Analysis, 1997, Vol. 11, pages 346-350.	1-27

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
10 June 1999	08 July 1999 (08.07.99)

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	SCHNASS e.h.

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No

PCT/US 99/03073

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARTIN, V. Immunomagnetische Anreicherung und Detektion metastasierender Tumorzellen. Bioforum, February 1996, Vol. 11, pages 494-496. --	1-27
A	EP 0395355 A1 (THE JOHNS HOPKINS UNIVER-SITY) 31 October 1990, claims. --	1-27, 79-89
A	US 5597531 A (LIBERTI et al.) 28 January 1997, claims. --	28-47
A	US 5698271 A (LIBERTI et al.) 16 December 1997, example 7. --	28-47
A	US 5512332 A (LIBERTI et al.) 30 April 1996, examples 9-11. ----	28-47

internationalen Recherchen-
.cht über die internationale
entanmeldung Nr.

to the International Search
Report to the International Patent
Application No.

au rapport de recherche inter-
national relatif à la demande de brevet
international n°

PCT/US 99/03073 SAE 225973

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im Recherchenbericht geführtes Patentdokument / Patent document cited in search report / document de brevet cité s le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets		Datum der Veröffentlichung Publication date Date de publication
A1 395355	31-10-1990	AT	E 100152	15-01-1994
		CA	AA 2014965	25-10-1990
		CA	C 2014965	15-09-1998
		DE	C0 69005885	24-02-1994
		DE	T2 69005885	28-04-1994
		DK	T3 395355	14-02-1994
		EP	B1 395355	12-01-1994
		ES	T3 2062352	16-12-1994
		JP	A2 3061480	18-03-1991
		US	A 5081030	14-01-1992
A 5597531	28-01-1997	AT	E 156366	15-08-1997
		CA	A1 1275533	23-10-1990
		DE	C0 3689618	17-03-1994
		DE	T2 3689618	11-05-1994
		DE	C0 3650644	11-09-1997
		DE	T2 3650644	12-03-1998
		EP	A1 244444	11-11-1987
		EP	A2 516198	02-12-1992
		EP	A3 516198	03-03-1993
		EP	B1 244444	02-02-1994
		EP	B1 516198	06-08-1997
		JP	T2 63501978	04-08-1988
		JP	B2 2588181	05-03-1997
		WO	A1 8702063	09-04-1987
		US	A 4795698	03-01-1989
		US	A 5108933	28-04-1992
		US	A 5512332	30-04-1996
		US	A 5866099	02-02-1999
		AT	E 137804	15-05-1996
		AU	A1 63403/90	03-04-1991
		CA	AA 2060182	23-02-1991
		DE	C0 69026949	13-06-1996
		DE	T2 69026949	28-11-1996
		EP	A1 489119	10-06-1992
		EP	A4 489119	01-07-1992
		EP	B1 489119	08-05-1996
		FI	A0 920763	21-02-1992
		JP	T2 5503188	27-05-1993
		WO	A1 9102811	07-03-1991
		US	A 5698271	16-12-1997
		CA	AA 2220169	19-12-1996
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		EP	A4 842042	28-10-1998
		WO	A1 9640502	19-12-1996
A 5698271	16-12-1997	US	A 5512332	30-04-1996
		AT	E 137804	15-05-1996
		AU	A1 63403/90	03-04-1991
		CA	AA 2060182	23-02-1991
		DE	C0 69026949	13-06-1996
		DE	T2 69026949	28-11-1996
		EP	A1 489119	10-06-1992
		EP	A4 489119	01-07-1992
		EP	B1 489119	08-05-1996
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		JP	T2 5503188	27-05-1993
		US	A 5597531	28-01-1997
		WO	A1 9102811	07-03-1991
		CA	AA 2220169	19-12-1996
		EP	A1 842042	20-05-1998
		EP	A4 842042	28-10-1998
		WO	A1 9640502	19-12-1996
		AT	E 156366	15-08-1997
		CA	A1 1275533	23-10-1990
		DE	C0 3689618	17-03-1994
		DE	T2 3689618	11-05-1994
		DE	C0 3650644	11-09-1997
		DE	T2 3650644	12-03-1998
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		EP	A2 516198	02-12-1992
		EP	A3 516198	03-03-1993
		EP	B1 244444	02-02-1994
		EP	B1 516198	06-08-1997
		JP	T2 63501978	04-08-1988
		JP	B2 2588181	05-03-1997
		WO	A1 8702063	09-04-1987
		US	A 4795698	03-01-1989
		US	A 5108933	28-04-1992

A	5512332	30-0█996	AT	E	156366	15-08-1997
			CA	A1	1275533	23-10-1990
			DE	C0	3689618	17-03-1994
			DE	T2	3689618	11-05-1994
			DE	C0	3650644	11-09-1997
			DE	T2	3650644	12-03-1998
			EP	A1	244444	11-11-1987
			EP	A2	516198	02-12-1992
			EP	A3	516198	03-03-1993
			EP	B1	244444	02-02-1994
			EP	B1	516198	06-08-1997
			JP	T2	63501978	04-08-1988
			JP	B2	2588181	05-03-1997
			WO	A1	8702063	09-04-1987
			US	A	4795698	03-01-1989
			US	A	5108933	28-04-1992
			US	A	55975331	28-01-1997
			US	A	5866099	02-02-1999
			AT	E	137804	15-05-1996
			AU	A1	63403/90	03-04-1991
			CA	AA	2060182	23-02-1991
			DE	C0	69026949	13-06-1996
			DE	T2	69026949	28-11-1996
			EP	A1	489119	10-06-1992
			EP	A4	489119	01-07-1992
			EP	B1	489119	08-05-1996
			FI	A0	920763	21-02-1992
			JP	T2	5503188	27-05-1993
			WO	A1	9102811	07-03-1991
			US	A	5698271	16-12-1997
			CA	AA	2220169	19-12-1996
			EP	A1	842042	20-05-1998
			EP	A4	842042	28-10-1998
			WO	A1	9640502	19-12-1996

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